Identification of protein binding sites involved in AChR localization at the developing neuromuscular junction

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January, 2007

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Your file Votre référence
ISBN: 978-0-494-32366-3
Our file Notre référence
ISBN: 978-0-494-32366-3

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Acknowledgements

I am most grateful to my mentor Dr. Michael Ferns for his guidance and patience during the course of my graduate studies. I am also thankful for his friendship and words of encouragements during difficult times. I have learnt an immense amount of science and feel privileged to have been his student for the past seven years.

I would also like to thank the members of my thesis committee: Drs. Salvatore Carbonetto and Paul Holland for their helpful advice over the course of this work. I thank Dr. Samuel David for being a co-supervisor and allowing me to graduate as a McGill student.

I was very fortunate to have Lucia, Jacinthe, Doreen, and John as my labmates during my time as a part of the Ferns lab. I also thank Drs. Sid Mitra and Sergey Yechikov, talented postdocs in the lab, for their technical help and insights. I also thank Jeff, Lorne, Rob, Scott and others that I have met the last three years at UC Davis, for their help in adjusting to a new surrounding. They have been like a family to me away from home and made my journey through graduate studies much more enjoyable.

Finally, I would like to thank my biggest supporter, my wife Hyejin for her love and encouragement. Thank you for always believing that I could get this done. I love you.

Rationale and Objectives

Motoneuron-derived agrin, signaling through muscle-specific kinase (MuSK), directs the precise localization of acetylcholine receptor (AChR) clusters underneath the nerve terminal (Kummer et al., 2006). Receptor localization is essential for synaptic transmission at the neuromuscular junction and involves direct and indirect interactions between the receptor and postsynaptic cytoskeletal and/or scaffolding proteins (Banks et al., 2003). In this thesis, I have investigated the protein interactions that contribute to synaptic AChR localization and how agrin signaling regulates these interactions.

Synaptic localization of AChR involves receptor anchorage to the cytoskeleton, since clustered receptors are less detergent extractable and less mobile in the plane of the muscle membrane than those that are diffusely distributed (Meier et al., 1995). AChR localization also requires rapsyn (Gautam et al., 1995) a muscle-specific scaffolding protein thought to bind and cluster the receptor at the synapse. However, it is presently unclear where rapsyn binds on the AChR and how rapsyn promotes receptor accumulation at the synapse. In addition, it is not known whether the AChR interacts with additional scaffolding and/or cytoskeletal proteins that also contribute to its synaptic localization. Finally, the agrin signaling events that modulate these protein interactions to produce efficient synaptic clustering of AChR are poorly understood.

The first objective of the thesis was to map the domain of the AChR that mediates agrin-induced clustering in muscle as an initial step in understanding the underlying molecular interactions that mediate synaptic AChR localization. My second objective was to determine rapsyn's site of interaction on the AChR. The next objective was to test whether agrin signaling modulates AChR-rapsyn interaction via β subunit phosphorylation. My final objective was to identify domains of rapsyn required to

mediate agrin/MuSK-induced AChR phosphorylation, which regulates its synaptic localization. Together, this study provides original insights on the protein interactions that mediate synaptic localization of the AChR at the vertebrate neuromuscular junction, and how this process is regulated by motoneuron-derived agrin.

Abstract

At the developing vertebrate neuromuscular junction (NMJ), the synaptic localization of the acetylcholine receptor (AChR) is regulated by a motoneuron-derived factor called agrin, and also requires an intracellular scaffolding protein called rapsyn. Rapsyn is thought to bind and cluster the AChR in the postsynaptic membrane; however, it is unclear where rapsyn binds on the AChR and if other proteins also contribute to receptor localization. To define the protein interactions that localize the AChR at the synapse, we utilized chimeric proteins consisting of CD4 fused to each of the AChR subunit large intracellular loops. First, by expressing the CD4-loop chimeras in cultured mytoubes, we identify a motif in the β subunit loop that is sufficient for agrin-induced clustering. Agrin induced rapid phosphorylation of a conserved tyrosine (Y390) in this motif and mutation of this site abolished clustering. Moreover, overexpression of the β-subunit motif inhibited agrin-induced clustering of the endogenous AChR. Next, by co-expressing rapsyn and the CD4-loop chimeras in heterologous cells, we show that rapsyn clusters and cytoskeletally anchors the large intracellular loop of the AChR α, β and ε subunits. This interaction was mediated by rapsyn binding to an α-helical domain conserved between the subunit loops, and occurred independent of agrin signaling in cultured myotubes. These findings suggest that at least two distinct interactions contribute to AChR localization at the NMJ: (constitutive) rapsyn binding to the α-helix of one or more subunit loops, and regulated binding of an additional protein to the β-subunit motif. As the second interaction is phosphorylation-dependent, it likely serves as an important regulatory step in the agrin signaling pathway localizing the AChR at the synapse.

In addition to its structural role, rapsyn is required for agrin-induced phosphorylation of the AChR. By expressing a series of rapsyn deletion mutants together with MuSK and AChR in heterologous cells, we find that deletion of just the rapsyn RING domain abolishes MuSK-induced AChR phosphorylation. Moreover, we find that the rapsyn C-terminal region is both necessary and sufficient for tyrosine kinase activation. Together, these findings suggest that rapsyn facilitates AChR phosphorylation by activating tyrosine kinases via its C-terminal domains.

Résumé

Au cours du développement de la jonction neuromusculaire (JNM), l'agrine orchestre la localisation des récepteurs à l'acétylcholine (AChRs) à la synapse. Ce processus nécessite non seulement ce facteur dérivé des motoneurones, mais aussi la présence d'une protéine de soutien nommé rapsyne. Cette dernière, localisée intracellulairement du côté postsynaptique, interagie directement avec les AChRs pour induire l'agglomération des AChRs. Toutefois, les domaines protéiques de la rapsyne impliqués dans cette interaction n'ont pas encore été identifié. Qui plus est, l'implication, d'autres protéines-clés dans ce processus reste encore inconnue. Pour tenter d'identifier les interactions protéiques nécessaires à la localisation des AChRs à la synapse neuromusculaire, nous avons créé des protéines chimères composées d'un domaine CD4 fusionné à chacune des boucles intracellulaires des sous-unités des AChRs (boucles-CD4). Tout d'abord, l'expression de ces constructions chimères par transfection de myotubes en culture nous a permis d'identifier un motif dans la boucle de la sous-unité b qui est suffisant pour permettre l'agglomération des AChRs en présence d'agrine. En effet, l'agrine induit une phosphorylation rapide d'un résidu tyrosine (Y390) dans la boucle de la sous-unité b des AChRs, et l'agglomération des AChRs est empêchée une fois ce site tyrosine muté. De plus, une surexpression de la boucle-CD4 de la sous-unité b empêche également l'agglomération des AChRs endogènes en présence d'agrine. Nous avons également co-exprimé la rapsyne ainsi que les différentes boucles-CD4 dans des cellules hétérologues et avons démontré que le rapsyne agglomère et lie au cytosquelette les sous-unités a, b et e. Cette interaction est rendue possible grâce au domaine a -hélicale qui est évolutivement conservé d'une sous-unité à l'autre. De plus, cette interaction est indépendante de la cascade de signalisation induite par l'agrine. Ces résultats suggèrent

donc qu'il existe 2 interactions distinctes contribuant à l'agglomération des AChRs à la membrane postsynaptique de la JNM : une interaction entre un ou des domaines a - hélicale des sous-unités des AChRs et la rapsyne, ainsi qu'une interaction régulée par l'agrine entre un domaine de la sous-unité b des AChRs et une protéine additionnelle non identifiée. Étant donné que la seconde interaction nécessite une phosphorylation de la sous-unité b par l'agrine, il est fort probable que cette interaction soit une importante étape régulatrice dans la cascade de signalisation induite par l'agrine lors de l'agglomération des AChRs à la synapse.

En plus de son rôle structural, la rapsyne est nécessaire lors du processus de phosphorylation des AChRs. En exprimant une série de mutation réductionnelle de la rapsyne en combinaison avec MuSK et les AChRs dans des cellules hétérologues, nous avons démontré que le domaine « RING » de la rapsyne est crucial au processus de phosphorylation des AChRs. Nous avons également découvert que la région de la terminaison-C de la rapsyne est nécessaire et suffisante pour induire l'activation d'une tyrosine kinase. L'ensemble de ces résultats suggère que la rapsyne facilite la phosphorylation des AChRs en activant une tyrosine kinase à l'aide de son domaine C-terminal.

Abbreviations

α-BuTX	α-bungarotoxin
aa	amino acid
ACh	acetylcholine
AChE	acetylcholinesterase
AChR	acetylcholine receptor
APC	adenomatous polyposis coli
ARIA	acetylcholine receptor-inducing activity
ChAT	choline acetyltransferase
DGC	dystrophin-associated glycoprotein complex
LN	long amino
MASC	myotube associated specificity component
MuSK	muscle specific tyrosine kinase
NMJ	neuromuscular junction
nNOS	neuronal nitric oxide synthase
NT3	neurotrophin 3
PAK1	p21-activated kinase
PDZ	PSD-95, Discs-large, Z01
PTB	phosphotyrosine binding
Rapsyn	receptor associated protein at the synapse
RATL	rapsyn associated transmembrane linker
RING	really interesting new genes
RTK	receptor tyrosine kinase
SH2	src homology 2
SN	short amino
TPR	tetratricopeptide repeat
wt	wild type

Contribution of authors

This thesis is comprised of a series of manuscripts with multiple authors. Below is a description of my contribution to each of them

Chapter II - Rapsyn interacts with the AChR via an a-helical motif conserved between the α, β, and ε subunit intracellular loops

Young Lee and Michael Ferns

This paper is completely my work. I generated the data for all the figures and co-wrote the paper.

Chapter III - A motif in the β subunit intracellular loop contributes to postsynaptic clustering of the acetylcholine receptor via a phosphorylation-dependent protein interaction

Lucia S. Borges, Sergey Yechikov, **Young Lee** and Michael Ferns

I generated the data for figures 5, 6 and 7, and co-wrote the paper.

Chapter IV - Rapsyn C-terminal domains mediate MuSK-induced phosphorylation of the AChR

Young Lee and Michael Ferns

This paper is completely my work. I generated the data for all the figures and co-wrote the paper.

Doctoral Publications

Peer reviewed papers

Borges, L.S., **Lee, Y.**, and Ferns, M. (**2002**) *Dual role for calcium in agrin signaling and acetylcholine receptor clustering.* **The Journal of Neurobiology. 50:69-79**

Borges, L.S., Yechikhov, S.Y., **Lee, Y.**, and Ferns, M. *A motif in the β subunit intracellular loop contributes to postsynaptic clustering of the acetylcholine receptor via a phosphorylation-dependent protein interaction.* (Submitted)

Abstracts

S. Yechikhov, L.S. Borges, **Y.I. Lee** and M.J. Ferns
The β subunit Intracellular Loop is Sufficient for Postsynaptic Clustering of the nAChR.
The American Society for Cell Biology 45th Annual Meeging
San Francisco, CA (December 10th-14th, 2005)

Y.I. Lee, L.S. Borges, and M.J. Ferns
Rapsyn interacts preferentially with the major intracellular loop of the nAChR β subunit.
Society of Neuroscience 32nd Annual Meeting
New Orleans, LA (November 8th-12th, 2003)

Y.I. Lee, S.L. Swope, and M.J. Ferns
Rapsyn's C-terminal Domain Mediates MuSK-Induced Phosphorylation of the AChR
The American Society for Cell Biology 42nd Annual Meeting
San Francisco, CA (December 14th-18th, 2002)

Chapter I

Literature Review

SYNAPTIC STRUCTURE AND FUNCTION AT THE MATURE NEUROMUSCULAR JUNCTION

The vertebrate neuromuscular junction is composed of parts of three cells: spinal motoneuron, skeletal muscle fiber, and terminal Schwann cell (Fig 1). The presynaptic nerve terminal and juxtaposed postsynaptic muscle membrane found at the mature neuromuscular junction are highly specialized structures that ensure fast and efficient chemical transmission mediated by the neurotransmitter acetylcholine (ACh).

The presynaptic nerve terminal of the motor neuron is a cellular structure specialized for release of the neurotransmitter (Hall and Sanes, 1993; Sanes and Lichtman, 1999). It contains a large number of 50 nm synaptic vesicles, which contain ACh, in addition to many mitochondria that are present to supply the energy required for synthesis and release of the neurotransmitter. The synaptic vesicles are often found docked at or concentrated around the active zones, sites at which synaptic vesicles fuse and release ACh.

In a complementary fashion, the postsynaptic muscle membrane innervated by the presynaptic nerve terminal is specialized to respond to release of ACh fast and without failure (Hall and Sanes, 1993; Sanes and Lichtman, 1999). The synaptic portion of the muscle membrane is found within shallow gutters of the muscle surface, on top of which the presynaptic nerve terminal is positioned. The synaptic gutters are further invaginated by secondary or postsynaptic folds, with their openings aligned precisely with the presynaptic active zones. There is a high-density accumulation of AChR at the top of the junctional folds (~10,000/μm^2 vs ~10/μm^2 in extrasynaptic areas of the muscle) while voltage-gated sodium channels are found concentrated at the bottom of the folds. Similarly, cytoskeletal components responsible for high-density accumulation of AChR and voltage-gated sodium channel are found in discrete domains of synaptic folds with

the appropriate ion channel. Such structural and molecular architecture of the postsynaptic apparatus at the vertebrate NMJ allows efficient translation of ACh release from presynaptic nerve terminal to excitatory endplate potentials, which in turn generate action potentials and subsequent muscle contractions.

The basal lamina, a layer of extracellular matrix material that ensheaths the entire muscle fiber, runs through the synaptic cleft (Sanes, 2003). Interestingly, the synaptic portion of the basal lamina differs in its composition to the extrasynaptic area, and contain numerous molecules that are required for proper formation and maturation of the NMJ (Patton, 2003). Moreover, the synaptic basal lamina also contains the acetylcholine esterase (AChE) that terminates synaptic transmission by hydrolyzing ACh (Sanes and Lichtman, 1999; Sanes, 2003).

Finally, a subset of glial cells called perisynaptic (or terminal) Schwann cells caps the nerve terminal and isolate the synapse from the environment, possibly enhancing efficiency of synaptic function (Kang et al., 2003; Koirala et al., 2003). In additional, these terminal Schwann cells, distinct from myelinating Schwann cells found in preterminal regions of the motor axon, are required at the site of nerve-muscle contact to stably maintain the presynaptic nerve terminal during maturation of the synapse.

DEVELOPMENT OF THE NEUROMUSCULAR JUNCTION

Synaptic localization of the AChR

The most prominent feature of a mature NMJ is the high-density aggregation of AChR in the muscle membrane. The proportion of the surface area occupied by the NMJ on each individual muscle fiber is only approximately 0.1% (Sanes and Lichtman, 1999). Yet, extremely high-density clusters of the AChR are formed and maintained in the

postsynaptic muscle membrane while the density of the receptor present in the extrasynaptic portions of the muscle is about 1000 fold less. How do muscle fibers produce and maintain these high-density aggregates of AChR during development? An exchange of signals between the motorneuron and the muscle fiber is required to induce differentiation of the pre- and postsynaptic specializations at the developing NMJ. While the identity of the molecules and sequence of events that lead to the synaptic clustering of the AChR are not yet been fully elucidated, these signals induce high-density accumulation of AChR by combination of an increased rate of local synthesis and membrane insertion, a decreased rate of local removal or degradation, and local immobilization.

Pre-patterning of muscle prior to innervation

Early *in vitro* studies showed that muscle cells in culture have the ability to form AChR clusters on surface independently of innervation (Anderson and Cohen, 1977; Anderson et al., 1977). These spontaneous clusters were, however, ignored by co-cultured motoneurons, which extended axons randomly over the muscle cells (Anderson and Cohen, 1977). Instead, motor axons induced new AChR clusters where they contacted the myocyte, and also dispersed the preformed clusters, with AChRs from the dispersed clusters becoming integrated into the ones formed by the neuron (Anderson and Cohen, 1977). Such experiments gave rise to the "neurocentric" model of NMJ formation (Fig 2A), where muscle plays the role of a naïve partner that merely provides the components required to assemble the postsynaptic specialization in response to nerve-derived synaptogenic cues. For the next two decades this perceived hierarchical relationship between nerve and muscle during synaptogenesis met little challenge as a

large body of *in vitro*, *in vivo*, and genetic studies, which identified key proteins that participate in the process, gave support to this hypothesis.

The challenge to the neurocentric view of the NMJ formation came initially from the analysis of mice lacking DNA isomerase-IIβ (Yang et al., 2000). Clusters of AChR were found in the mid regions of muscle in these mice despite the apparent lack of innervation. In addition, a mouse in which the motor neurons were genetically eliminated showed that these aneural AChR clusters also persist in absence of innervation (Lin et al., 2001; Yang et al., 2001). The prepatterning of the muscle also occurred transcriptionally. *In situ* hybridization results show that AChR α subunit messenger RNA is concentrated in the central region of aneural muscle (Lin et al., 2001; Yang et al., 2001). Furthermore, the preformed AChR clusters can "mature" and differentiate into complex "pretzel" shapes in culture the in absence of a presynaptic partner (Kummer et al., 2004), much like the mature NMJ *in vivo*, and led the authors to speculate that muscle may shape the nerve terminal arbors at the NMJ. The propensity of muscle fibers to undergo autonomous "postsynaptic" differentiation demonstrates that skeletal muscle is not the naïve postsynaptic partner long perceived to be. It is not yet clear whether the prepattering of the muscle is critical for formation and maturation of NMJ during development. However, a pair of recent studys (Flanagan-Steet et al., 2005; Panzer et al., 2005) demonstrated that at least a proportion of the muscle autonomous AChR clusters are recognized by the motor axons during innervation and utilized to form synapses (Fig 2B).

Nerve-derived factors that shape the developing NMJ: ACh and agrin

So, what role does the motor neuron play in the formation of the NMJ during development? Although the muscle is prepatterned, the nerve provides signaling molecules required to stably cluster the AChR specifically at the synapse (Burden, 2002; Kummer et al., 2006). Two such nerve-derived factors discussed in detail here are agrin and ACh.

Agrin – motor neuron derived AChR clustering agent

Agrin, a heparan-sulfate proteoglycan with an approximate molecular weight of 450kDa, was isolated initially from *Torpedo californica* electric organ based on its activity to induce high-density aggregation of AChR in muscle (Nitkin et al., 1987). Multiple agrin isoforms are generated through alternative splicing of agrin transcript at both the N- and C-termini. Incorporation of one of two alternative exons at the 5' end of agrin transcript produces agrin isoforms with distinct subcellular distributions: LN (long-amino) and SN (short-amino) (Burgess et al., 2000; Neumann et al., 2001). The LN agrin, containing a unique 150 amino acid stretch encoded by the first three exons of the long transcript, is expressed and secreted by many different tissues, including skeletal muscle and motor neurons (Burgess et al., 2000). The 150 amino-acid portion of LN agrin binds to laminin and allows agrin to be incorporated into the basal lamina (Denzer et al., 1997). The first exon of the short transcript (SN-agrin) encodes for a 49 amino acid fragment, which converts agrin into a type II transmembrane protein with an intracellular N-terminus and an extracellular C-terminus, and is found only in neurons (Burgess et al., 2000; Neumann et al., 2001). Moreover, the expression of the two isoforms is thought to be driven by separate promoters, consistent with the distinct tissue distribution of the two N-terminal splice variants (Burgess et al., 2000).

Differential splicing of agrin transcript near its C-terminus generates isoforms with different AChR clustering activities (Ferns et al., 1992; Ruegg et al., 1992), occurs in a cell-specific manner and only neural tissue expresses the isoforms of agrin active in inducing the aggregation of AChR. It is the inclusion of an eight amino-acid insert at the Z-splice site that confers the Z+ (neural) isoform of agrin the ability to cluster the AChR in the postsynaptic muscle membrane with approximately 10,000 fold greater efficiency than the Z- (non-neural) isoforms of agrin, which lack the eight amino-acid inserts (Ferns et al., 1992; Ferns et al., 1993). Therefore, alternative splicing of agrin transcript appears to be regulated in a tissue specific manner and determines agrin's subcellular distribution as well as it AChR clustering activity.

Numerous studies have demonstrated Z+ agrin's ability to induce clustering of the AChR. However, the most convincing evidence for agrin's requirement for the formation of mammalian NMJ came from the generation of neural agrin null (Z+ agrin -/-) mice (Gautam et al., 1996; Burgess et al., 1999). At birth, the agrin knockout mice had severely impaired pre- as well as post-synaptic differentiation at the NMJ. Many post-synaptic components such as the AChR, AChE, erbB3, laminin β2, rapsyn, and utrophin fail to become localized at the nerve/muscle contacts. Although a small degree of pre- and post- synaptic specializations do occur in Z+ agrin null mice, the myoneural contacts invariably fail to form functional synapses and the mutant animals die at birth due to respiratory failure. A full agrin knockout mouse, which lacks all isoforms of agrin, displays a phenotype that is identical to that seen in the Z+ agrin null mice (Burgess et al., 2000). This suggests that muscle (Z-) agrin does not contribute significantly during neuromuscular synaptic differentiation. In addition, mice that specifically lack the LN isoforms of agrin also fail to form functional NMJ (Burgess et al., 2000). The various

knockouts of agrin, thus, indicate that the LN Z+ isoform of agrin is produced by spinal motor neurons and act to organize synaptic differentiation at NMJ during development.

ACh – a negative modulator of AChR clustering at the developing NMJ

The neurotransmitter, ACh, plays a role in maturation of the NMJ through activity-driven suppression of extrajunctional AChR expression. Postsynaptic blockade of synaptic transmission at NMJ using antagonists of AChR induces acetylcholine hypersensitivity, resulting from expression of AChR at extrajunctional regions of the muscle (Berg and Hall, 1975). In contrast, nerve-evoked activity or direct stimulation of muscle prevents or even reverses the ACh hypersensitivity by suppressing the expression of AChR in extra-synaptic regions (Lomo and Rosenthal, 1972). Analysis of mutant mice lacking expression of Choline acetyltransferase (ChAT, the only enzyme responsible for the synthesis of ACh) provide evidence that neurotransmission also plays a key role in early development of the NMJ. In addition to the excessive branching of the nerve terminal at the synapse, there were many ChAT knockout muscle fibers that carried more than one NMJ (Misgeld et al., 2002). Moreover, at late embryonic stages, NMJs found in the ChAT -/- seem to show advanced rate of maturation when compared to those found in the wild type (Misgeld et al., 2002). These findings suggest that neurotransmission at the NMJ has restrictive effects on its postsynaptic differentiation.

Opposing effects of agrin and ACh at the developing NMJ

Initial analysis of agrin knockouts (Z+ and full) were performed prior to the discovery of muscle prepatterning, and led to the conclusion that AChR clustering did not occur in absence of motor neuron-derived agrin (Gautam et al., 1996; Burgess et al.,

1999; Burgess et al., 2000). Upon closer observation, however, AChR was indeed found clustered in agrin -/- muscles early in embryonic development (E14-16)(Lin et al., 2001). The AChR clusters, however, start to disperse with the arrival of motor neurons and disappeared by birth (Lin et al., 2001). Moreover, muscle autonomous clusters persist till birth when innervation is prevented by genetically eliminating motor neurons (Lin et al., 2001; Yang et al., 2001). These finding suggested that agrin is required to stably cluster the AChR at the NMJ, and that it counteracts the effects of an additional nerve-derived signal(s) that disperses preformed AChR clusters.

The restrictive effects of neurotransmission on the developing NMJ (Misgeld et al., 2002) suggested that ACh might be the factor responsible for nerve-induced dispersal of preformed AChR aggregates. Consistent with this hypothesis, mice lacking both agrin and ChAT formed NMJs whose pre- and post-synaptic partners differentiated and persisted until birth (Lin et al., 2005; Misgeld et al., 2005). In fact, application of α-bungarotoxin, which blocks the binding of ACh to its receptor, prevented the dispersal of AChR aggregates that occur when co-cultured with agrin null motor neurons (Misgeld et al., 2005), while treatment of myotubes in culture with an analog of ACh, carbacholamine, which activates the AChR resulted in dispersal of spontaneous clusters (Lin et al., 2005; Misgeld et al., 2005). These results strongly suggest that neurotransmission is responsible for dispersal of preformed AChR clusters in the postsynaptic muscle fibers, and that agrin acts to stabilize AChR clusters by counteracting the anti-synaptogenic effects of neurotransmission.

While agrin seemed dispensable for postsynaptic differentiation when neurotransmission was abolished, there are notable postsynaptic defects in agrin/ChAT double knockout mice. First, some of the muscle fibers in the agrin/ChAT double

knockout animals lacked AChR clusters altogether while many others had multiple NMJs (Misgeld et al., 2005). Next, the number of high-density AChR aggregates in agrin-/-;ChAT-/- mice was about 25-30% of control or ChAT null mice (Lin et al., 2005). Moreover, it was previously shown that injection of cDNA encoding recombinant Z+ agrin into extra-junctional areas of muscle fiber *in vivo* induced many aspects of a mature NMJ at the precise location of recombinant Z+ agrin deposition (Cohen et al., 1997; Jones et al., 1997; Meier et al., 1997). So, while the data indicate that neural agrin stabilizes preformed AChR clusters, it also likely contributes to postsynaptic differentiation by initiating formation of new AChR clusters during normal development.

Postsynaptic components involved in AChR localization

MuSK

MuSK is a receptor tyrosine kinase (RTK) expressed in differentiated muscle fibers (Valenzuela et al., 1995; Glass et al., 1996), where it is synaptically localized with the AChR (Valenzuela et al., 1995; Bowen et al., 1998). The fact that MuSK knockout mice displayed severe neuromuscular defects that resemble those seen in the agrin null mice (DeChiara et al., 1996) suggested that MuSK may be the agrin receptor in muscle. Several other studies support the view that MuSK mediates agrin's ability to induce postsynaptic differentiation. First, unlike the agrin mutants, myotubes cultured from MuSK knockout animals fail to form AChR clusters either spontaneously or in response to agrin stimulation (Glass et al., 1996). While this defect is rescued by reintroducing MuSK into the MuSK null myotubes, expression of MuSK whose kinase domain is inactivated did not confer MuSK knockout myotubes with the ability to respond to agrin (Zhou et al., 1999; Herbst and Burden, 2000). Kinase-defective MuSK, in fact, acted in a

dominant-negative fashion to inhibit agrin-induced AChR clustering when over-expressed in wildtype muscle (Glass et al., 1997). Next, MuSK can be chemically cross-linked to agrin on muscle membrane (Glass et al., 1996), suggesting a close physical association between the two proteins. In addition, agrin application causes MuSK activation, as indicated by its rapid phosphorylation (Glass et al., 1996). Importantly, only the Z+ isoform of agrin, which stimulates clustering of AChR in muscle, causes MuSK to be activated (Glass et al., 1996). While cross-linking experiment suggests that MuSK activation involves agrin binding, direct interaction between the two proteins has not been demonstrated. Purified agrin does not bind to isolated MuSK ectodomain, or activate MuSK expressed in heterologous cells (Glass et al., 1996). Altogether, the evidence suggests that MuSK is the signaling component of the agrin receptor complex, while a co-receptor, tentatively named MASC (myotube-associated specificity component), is required for agrin binding (Glass et al., 1996).

Interestingly, unlike in agrin null mice, there are no AChR clusters on muscle fibers of MuSK null mice at any stage of development, even prior to the arrival of motor axons (Lin et al., 2001). Therefore, MuSK is absolutely required for AChR to cluster. In addition, this finding suggests that there may be agrin-independent modes of MuSK activation. Consistent with this idea, several treatments can activate MuSK in the absence of agrin. Experiments that used antibodies or a mutated transmembrane domain of MuSK to dimerize MuSK produced activation of MuSK and AChR clustering (Xie et al., 1997; Hopf and Hoch, 1998; Jones et al., 1999). Elevated extracellular Ca^{++} concentration or neuraminidase treatment of cultured myotubes resulted in similar MuSK activation and clustering of AChR (Grow et al., 1999). Moreover, overexpression of

MuSK at an extrasynaptic region of the muscle fiber produced AChR clusters similar to the ones induced by ectopic expression of agrin (Jones et al., 1999).

Distinct structural and functional domains of MuSK are critical for agrin-induced AChR clustering in muscle. Domain analysis using different extracellular deletion mutants showed that the extreme amino-terminal region is required for agrin-induced MuSK activation, presumably through its interaction with agrin and/or the co-receptor, MASC (Zhou et al., 1999). Studies also showed that the MuSK tyrosine kinase domain is required for AChR clustering as MuSK mutants with either deleted (Zhou et al., 1999) or inactive kinase domain (Herbst and Burden, 2000) failed to produce spontaneous or agrin-induced AChR aggregates. Remarkably, the kinase domain of another RTK, TrkA, can substitute that of MuSK(Herbst and Burden, 2000). However, when the entire intracellular domain of MuSK, the juxtamembrane region in addition to the kinase domain, is replaced with that of TrkA, the resulting chimeric receptor did not produce AChR clustering when activated by agrin (Herbst and Burden, 2000). In fact, a 13 amino acid motif from MuSK juxtamembrane region, that contains a PTB protein binding site (NPXY), is sufficient to confer the synaptogenically inactive MuSK/TrkA chimeric receptor the ability to produce agrin-induced AChR clusters (Herbst and Burden, 2000). The tyrosine within the PTB protein binding motif (Y553) is phosphorylated with agrin treatment and this requires MuSK kinase activity (Herbst and Burden, 2000). The mutation of this tyrosine abolishes MuSK's ability to induce AChR clusters (Zhou et al., 1999; Herbst and Burden, 2000). This suggests that the intracellular juxtamembrane region of MuSK is critical for translating agrin-induced MuSK activation to local signaling events that ultimately result in synaptic clustering of the AChR, and the kinase domain of MuSK is required to phosphorylate the juxtamembrane tyrosine. Quite

surprisingly, while the intracellular region of MuSK is required for agrin-induced AChR clustering, it is not sufficient. A reverse chimeric receptor TrkC/MuSK, where a corresponding region of TrkC replaces the extracellular region of MuSK, was not sufficient to induce AChR clustering in cultured myotubes despite being activated by the surrogate ligand NT3 (Glass et al., 1997) (but see (Sander et al., 2001)). The ectodomain of MuSK, then, performs functions that are pertinent to post-synaptic differentiation in addition to its interaction to agrin and/or MASC. Therefore, MuSK requires the "activities" of both the intracellular and extracellular domains for assembly of post-synaptic apparatus.

Rapsyn (the protein formerly known as the 43K protein)

Like most membrane proteins, AChR is free to diffuse within the plane of the muscle membrane prior to clustering (Axelrod et al., 1976); therefore, synaptic accumulation of AChR at the muscle endplate likely involves a mechanism that anchors the receptor to the synaptic cytoskeleton. Electron microscopic examination revealed a distinct "bar" associated with the intracellular face of *Torpedo* electric organ membranes (Sealock, 1982). Brief alkaline treatment of Torpedo membranes at pH 11, which removes this intracelluar electron-dense material(s) from the postsynaptic membrane fragments, significantly increased the rotational and lateral mobility of the AChR in the membrane (Lo et al., 1980; Cartaud et al., 1981; Rousselet et al., 1982). Another study showed that filamentous structures, presumed to be cytoskeletal elements, were found to be either attached or in close proximity to the postsynaptic domain of the Torpedo electrocyte membrane (Cartaud et al., 1981). These findings supported the idea that a

major component of the post-synaptic apparatus interacts with the AChR to crosslink and/or anchor it to the synaptic cytoskeletal network.

Fractionation of purified AChR-rich membranes of *Torpedo* electric organs identified a 43K protein as a major constituent that is present in approximately equimolar amounts with the AChR (Burden et al., 1983; LaRochelle and Froehner, 1986). The abundance of the 43K protein raised the possibility that it may be an element of the postsynaptic apparatus important for clustering of the AChR. Subsequent biochemical and immunohistochemical studies suggested that the 43K protein is a cytoplasmic membrane-associated postsynaptic protein (Froehner et al., 1981; St John et al., 1982; Porter and Froehner, 1983). Electron microscopy aided by monoclonal antibodies to the AChR and the 43K protein demonstrated that they co-localize at the crest of the synaptic folds in the Torpedo electric organ, and confirmed that 43K protein is found on the cytoplasmic side of the postsynaptic membrane (Sealock et al., 1984). Interestingly, alkaline extracts of the purified AChR-rich membrane from electric rays are composed primarily of the 43K protein (Froehner et al., 1981; Walker et al., 1984). In addition, the 43K protein binds to actin in an overlay experiment (Walker et al., 1984), demonstrating its ability to interact directly with the cytoskeleton. Therefore, since alkaline treatment removes the 43K protein from AChR-rich membrane, and, possibly as a result, renders the AChR more mobile in the plane of the membrane, the above findings suggested that 43K might interact with and directly anchor the AChR to the cytoskeleton.

In *Torpedo* electric rays, the 43K protein is expressed in skeletal muscle as well as its electric organ (LaRochelle and Froehner, 1986). In addition, immunohistochemical studies showed that the 43K protein colocalizes with AChR clusters at mammalian NMJs during all stages of development (Froehner et al., 1981; Porter and Froehner, 1983;

Burden, 1985; Noakes et al., 1993). The mouse homolog of Torpedo 43K protein was subsequently cloned and the protein was re-named receptor-associated protein at the synapse (RAPsyn) (Frail et al., 1988). Rapsyn transcript was found concentrated at synaptic sites in muscle fibers (Moscoso et al., 1995a), suggesting that the rapsyn gene is transcribed specifically by synaptic nuclei. In addition, transcripts encoding rapsyn and AChR subunits appear coordinately during embryonic development, and both are upregulated in denervated adult muscles (Baldwin et al., 1988). Therefore, these findings demonstrate a close spatial and temporal relationship between AChR and rapsyn during development, and is consistent with the idea that rapsyn is responsible for anchoring and/or clustering the AChR at the NMJ.

More recently, the study of rapsyn null mutant mice has confirmed that rapsyn is critical for the formation of functional mammalian NMJ. As seen in knockout mutants of agrin and MuSK, rapsyn knockout (rapsyn -/-) animals show severe defects in neuromuscular synaptic differentiation, and fail to form detectable AChR clusters at myoneural contacts (Gautam et al., 1995). Moreover, while agrin treatment activates MuSK in rapsyn null myotubes, it does not induce formation of AChR clusters (Apel et al., 1997). Therefore, these studies identify rapsyn as a critical downstream effector molecule that is indispensable for the agrin-induced AChR localization in the post-synaptic membrane at the NMJ.

How does rapsyn cluster the AChR specifically at the synapse? Remarkably, rapsyn forms surface-associated clusters when expressed in heterologous cells (Froehner et al., 1990). In addition, rapsyn is able to recruit surface AChR into such clusters (Froehner et al., 1990; Phillips et al., 1991b), suggesting a direct AChR-rapsyn interaction. However, the biochemical evidence for direct interaction between the two

proteins is yet to be produced. Unlike in heterologous cells, rapsyn and AChR do not cluster in muscle prior to agrin-induced MuSK activation. This suggests that there may be default inhibitory processes that prevent rapsyn-mediated AChR clustering, and that agrin signaling overrides this inhibition. Therefore, rapsyn likely interacts with additional postsynaptic components in order to cluster the AChR downstream of MuSK activation.

Studies in heterologous expression systems have demonstrated that rapsyn also interacts with β-dystroglycan (Apel et al., 1995; Cartaud et al., 1998) and MuSK (Gillespie et al., 1996; Apel et al., 1997). MuSK is concentrated at the sites of myoneural contact even in rapsyn null muscle (Apel et al., 1997). This rapsyn-independent localization of MuSK to the nerve/muscle contact suggests that MuSK is part of a primary scaffold at the nascent synapse, to which AChR is recruited in a rapsyn-dependent manner. Interestingly, this MuSK-rapsyn interaction requires the ectodomain of MuSK (Apel et al., 1997; Zhou et al., 1999), and must be mediated by a ubiquitous transmembrane protein, rapsyn-associated transmembrane linker (RATL) (Apel et al., 1997). This finding is consistent with the fact that extracellular region of MuSK is essential to produce AChR aggregation, and suggests a structural function for MuSK, in addition to its role as the signaling agrin receptor, during the formation of the NMJ. Rapsyn's interaction with β-dystroglycan (Apel et al., 1995; Cartaud et al., 1998), and actin (Walker et al., 1984) offers two possible ways in which the AChR can be anchored to the post-synaptic cytoskeleton: direct and indirect. A direct connection between the AChR and the cytoskeletal network would occur via rapsyn binding to cytoskeletal actin. Alternatively, indirect anchoring of the receptor could occur through the dystrophin-

associated glycoprotein complex (DGC) aided by rapsyn's ability to interact with β-dystroglycan.

Analysis of rapsyn's primary amino-acid sequence predicts the existence of several functionally important structural motifs (Fig 3). While the three-dimensional structure of rapsyn remains undetermined, a series of recent tests of rapsyn mutants in heterologous expression systems has shown that rapsyn's ability to be targeted to the membrane, to self-aggregate, and to cluster components of the post-synaptic apparatus are attributed to distinct and separate functional domains. First, at the N-terminal end of the protein, rapsyn contains a covalently bound myristate, a fatty-acid modification (Musil et al., 1988; Carr et al., 1989b). Elimination, masking, or mutation of the N-terminus myristoylation consensus sequence prevents the targeting of the protein to the cell surface (Phillips et al., 1991a; Ramarao and Cohen, 1998). The protein is also predicted to have: eight tetratricopeptide repeats (TPRs)(Ponting and Phillips, 1996; Ramarao and Cohen, 1998), a leucine-zipper (Froehner, 1991), a possible coiled-coil structure (Ramarao and Cohen, 1998), and a Zn^{++} binding RING structure (Froehner, 1991; Scotland et al., 1993). Studies suggest that the TPRs within the N-terminal portion are critical for rapsyn to self-aggregate (Ramarao and Cohen, 1998; Ramarao et al., 2001; Eckler et al., 2005). In contrast, the C-terminal portions of the protein are required for association with some of the known components of the post-synaptic apparatus. The coiled-coil domain and the RING structure near the C-terminal end of rapsyn are required for the protein's ability to associate with and cluster the AChR (Ramarao and Cohen, 1998; Ramarao et al., 2001), and β-dystroglycan (Bartoli et al., 2001), respectively. In addition, possible phosphorylation sites at serine 406 (Froehner, 1991) and tyrosine 362 (Mohamed and

Swope, 1999) are predicted; raising the possibility rapsyn may be phosphorylated and may explain the charge heterogeneity of rapsyn observed during its initial characterization by two-dimensional electrophoresis (Gysin et al., 1981; Porter and Froehner, 1983; Sealock et al., 1984). It is suspected that rapsyn interacts with additional postsynaptic components, but further studies would be required identify the interacting partners and rapsyn domains responsible for these proposed interactions.

A pair of recent studies reported the presence of rapsyn transcripts in neuronal tissue (Burns et al., 1997; Feng et al., 1998); raising the possibility that rapsyn may be involved in clustering of neuronal AChRs at fast cholinergic interneuronal synapses. Moreover, rapsyn clusters neuronal AChRs in heterologous cells (Feng et al., 1998; Kassner et al., 1998). However, while rapsyn transcript is present in some neuronal tissues, rapsyn protein is not detected in tissues other than skeletal muscle (Musil et al., 1989; Feng et al., 1998). Second, the interactions between rapsyn and neuronal AChRs in heterologous cells occur intracellularly and not on the cell surface (Feng et al., 1998), raising questions about their physiological relevance. Lastly, clusters of neuronal AChR are present in superior cervical ganglia of rapsyn null mice (Feng et al., 1998). Therefore, results show that while rapsyn can interact with neuronal AChRs, it is not required for clustering of neuronal AChRs at interneuronal synapses in mouse sympathetic ganglia.

Although synaptic localization of the AChR at the NMJ is absolutely dependent on rapsyn, several recent studies show that reverse is also true and suggest that the neurotransmitter receptor is required for the synaptic localization of rapsyn and other post-synaptic proteins. AChR-dependent synaptic localization of rapsyn (Ono et al., 2001) is likely mediated by direct interaction between the two. Furthermore, co-immunoprecipitation of rapsyn with unclusterd AChR from extrasynaptic areas of

denervated muscle (Moransard et al., 2003), and the finding that two proteins are co-targeted through the secretory pathway to the cell surface (Bignami et al., 1998; Marchand et al., 2000; Marchand et al., 2002; Moransard et al., 2003) suggest that the AChR-rapsyn interaction occurs prior to agrin signaling, and perhaps even surface expression of the two.

While the C-terminal coiled-coil domain of rapsyn mediates its interaction with the AChR (Ramarao and Cohen, 1998; Ramarao et al., 2001), the reciprocal site of interaction on the receptor is not defined. AChR is composed of five homologous subunits ($\alpha 2\beta\gamma\delta$ for fetal form and $\alpha 2\beta\varepsilon\delta$ for mature form; reviewed in (Lindstrom, 2003)). All of the subunits have the same membrane topology: extracellular N- and C-termini, four transmembrane domains (M1 through M4), and a large intracellular loop between the third and the fourth transmembrane domains where AChR interacting proteins most likely bind. Consistent with this idea, untrastructural studies using electron microscopy show a non-receptor protein in close association with the cytoplasmic portion of the AChR (Miyazawa et al., 1999). This abundant receptor associated protein is removed by alkaline extraction (Toyoshima and Unwin, 1988), suggesting that it is rapsyn. The fact that rapsyn and the β subunit of the AChR can be chemically cross-linked (Burden et al., 1983) suggests that the association seen between rapsyn and AChR might be mediated by the β subunit of the neurotransmitter receptor. In addition, a modified yeast-two hybrid experiment suggests that the AChR β subunit and rapsyn interact directly (Bartoli et al., 2001). However, a pair of studies reports that AChR subunits other than the β subunit are able to interact with rapsyn (Maimone and Merlie,

1993; Huebsch and Maimone, 2003). Therefore, the identity of the AChR subunit(s) responsible for mediating the association with rapsyn is still in debate.

Dystrophin-glycoprotein complex

The dystrophin-glycoprotein complex (DGC), whose major components are dystrophin/utrophin, dystroglycans, α-dystrobrevins, and syntrophins, is found throughout the surface of the muscle membrane and functions as a membrane-spanning molecular linker between the basal lamina and the actin cytoskeleton of muscle, providing mechanical stability to the muscle fibers (Durbeej and Campbell, 2002; Lapidos et al., 2004). Accordingly, mutations of many of its components lead to muscular dystrophies of varying severity. Components of the DGC complex are also concentrated at the NMJ, and multiple lines of evidence indicate that they contribute to the formation of the NMJ (Banks et al., 2003). Knock-out studies of DGC complex components demonstrated, however, that they are not required for the initial clustering of the AChR; instead, they seem to be required for the maturation and/or the stability of the synaptic AChR aggregates (Banks et al., 2003).

Dystroglycan

Posttranslational processing of a single dystroglycan gene product yields two non-covalently bound proteins: the extracellular α- and the transmembrane β-dystroglycan (Ibraghimov-Beskrovnaya et al., 1992; Deyst et al., 1995). Dystroglycans provide a transmembrane bridge that extends from the muscle basal lamina to the submembrane cytoskeleton via α-dystroglycan's interaction with laminin (Ibraghimov-Beskrovnaya et

al., 1992; Ervasti and Campbell, 1993; Deyst et al., 1995), and β-dystroglycan's intracellular interaction with utrophin/dystrophin (Jung et al., 1995).

Dystroglycan's concentration at the NMJ (Matsumura et al., 1992) as well as the ability to bind agrin (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994) were the initial indications that dystroglycan is involved in neuromuscular synapse formation. However, α-dystroglycan binds both nerve- and muscle-derived agrin (Sugiyama et al., 1994; Gesemann et al., 1996), and domains of agrin responsible for AChR aggregation and interaction with α-dystroglycan are distinct (Gesemann et al., 1996; Hopf and Hoch, 1996; Jacobson et al., 1998). Moreover, α-dystroglycan is dispensable for agrin-induced activation of MuSK (Jacobson et al., 1998). These findings indicate that dystroglycan is not required for agrin/MuSK signaling in muscle.

Regardless, dystroglycan is crucial for normal organization of AChR clusters at the NMJ. Agrin-induced AChR clusters found on the dystroglycan null myotubes were fragmented, less dense and about three times the size of the AChR clusters in wildtype myotubes (Grady et al., 2000; Jacobson et al., 2001). Distribution of the AChR was similarly perturbed at the NMJs of the dystroglycan-null chimeric mice (Cote et al., 1999). These results are consistent with earlier findings where α-dystroglycan antibody induced fragmentation of AChR clusters on cultured myotubes (Campanelli et al., 1994; Gee et al., 1994). In addition, AChR clusters found on the null myotubes were less stable as they dispersed faster upon agrin withdrawal compared to the ones found on the wildtype myotubes (Jacobson et al., 2001). Therefore, these findings indicate that dystroglycan is critical for maturation and stability of the AChR clusters, rather than their initial formation.

Dystrophin/utrophin

Dystrophin is found at the troughs of the synaptic folds along with the voltage gated sodium channels, whereas its homologue, utrophin, is colocalized with AChR at the top of the junctional folds (Bewick et al., 1992). Both dystrophin and utrophin are able to interact with actin and β-dystroglycan, via its N- and C-termini respectively (Ward and Davies, 1998). Therefore, utrophin can interact indirectly with synaptic AChR at the top of the junctional folds via its interaction with β-dystroglycan, which in turn binds rapsyn (Cartaud et al., 1998; Bartoli et al., 2001), and anchored the receptor to the synaptic cytoskeleton. Utrophin is found concentrated at the AChR-rich domains from early stages of AChR clustering (Phillips et al., 1993; Campanelli et al., 1994; Gee et al., 1994), and overexpression of utrophin fragment prevents AChR clustering in cultured myotubes (Namba and Scheller, 1996).

While these findings implicate utrophin in postsynaptic differentiation, mice lacking utrophin show surprisingly mild defects at the NMJ including a reduction in the number of junctional folds by ~50% compared to wildtype NMJs (Deconinck et al., 1997b; Grady et al., 1997a), and a 30% decrease in the AChR density at the synapse (Deconinck et al., 1997b; Grady et al., 1997a). In contrast, the absense of utrophin did not alter the synaptic localization of cytoskeletal proteins rapsyn, β2-syntrophin, β-dystroglycan, and α-dystrobrevin (Deconinck et al., 1997b; Grady et al., 1997a).

To determine whether dystrophin could compensate for the lack of utrophin expression, similar analysis was performed in double null mice. NMJs of mice lacking both dystrophin and utrophin displayed more pronounced defects in the morphology of

AChR aggregates than those found in the utrophin knockout animals. NMJs of the double deficient animals were divided into multiple boutons (Grady et al., 1997b), possibly due to repeated regeneration of the muscle fiber, and there was a further reduction in the number of junctional folds compared to the utrophin null animals (Deconinck et al., 1997a; Grady et al., 1997b). In addition, the double knock-out NMJ displayed a significant reduction in the levels of β2-syntrophin, β-dystroglycan, and α-dystrobrevin (Grady et al., 1997b). The exaggerated phenotype seen at the NMJs of the double knockout mice suggest that dystrophin compensated for the lack of utrophin. These findings suggest that, like dystroglycan, utrophin is not required for initial clustering of the AChR at the NMJ, but instead for maturation of the synapse and formation of the junctional folds.

Dystrobrevin

Dystrobrevin was initially purified as a major tyrosine phosphorylated protein associated with AChR-rich membranes of *Torpedo* electric organ (Carr et al., 1989a; Wagner et al., 1993). Alternative splicing of α-dystrobrevin transcript gives rise to five isoforms, three of which are expressed in skeletal muscle: α-dystrobrevin 1-3 (Enigk and Maimone, 2001). The distribution of α-dystrobrevin-1 resembles that of utrophin and is found enriched at the top of the junctional folds with AChR and rapsyn, while α-dystrobrevin-2 is found mostly at the bottom of the folds and at extrasynaptic sites, a cellular localization that mirrors that of dystrophin (Bewick et al., 1992; Peters et al., 1998). NMJs of mice lacking α-dystrobrevin show an abnormal morphology where AChR clusters have a frayed appearance compared to the smooth borders seen at

wildtype NMJs (Grady et al., 2000). The number of junctional folds was reduced by approximately 50% compared to wildtype animals, and the AChR was no longer restricted to the crest of the remaining junctional folds (Grady et al., 2000). There is a ~70% reduction in the AChR density at the NMJ, which may be explained, at least in part, due to the increased turnover of the receptor in the mutant animals (Akaaboune et al., 2002). Stability of AChR clusters also seems compromised in the absence of α-dystrobrevin, as null myotubes show a reduction in the number of agrin-induced AChR clusters as well as an accelerated rate of AChR cluster dispersal upon agrin-withdrawal compared to the those found in wildtype myotubes (Grady et al., 2000).

Syntrophin

Identified initially from AChR-rich membranes of *Torpedo* electric organ (Froehner, 1984), syntrophins are modular proteins made up of several functional protein motifs (Adams et al., 1995; Ahn et al., 1996): two plecstrin homology domains, a PDZ domain and a C-terminal syntrophin-unique domain. Syntrophins' ability to bind to dystrobrevin and dystrophin/utrophin (Kramarcy et al., 1994; Ahn and Kunkel, 1995; Ahn et al., 1996) as well as to signaling molecules such as nNOS (Brenman et al., 1996) suggests that syntrophins function as molecular adaptors that recruit signaling proteins to the DGC.

While three members of the syntrophin family of proteins are found at the NMJ α1, β1, and β2), their distribution and expression pattern differ (Kramarcy and Sealock, 2000), suggesting subtle differences in how each contributes to the formation of the NMJ. The roles of syntrophins in development of the NMJ were tested in mice lacking different

syntrophins. While mice lacking α-syntrophin displayed no obvious behavioral deficit, the NMJs of these mice were severely altered at both structural and molecular levels (Adams et al., 2000). Structurally, the α-syntrophin -/- NMJs have shallow synaptic gutters as well as a reduction in the number of secondary folds that are open to the cleft. AChR present at the synapse was reduced to ~35% of that seen in wildtype endplates, and the remaining AChR clustered at the synapse showed frayed and irregular appearance as opposed to the smooth borders in wildtype muscle. The α-syntrophin null NMJs also lacked utrophin and nNOS. While mice lacking β2-syntrophin had no detectable phenotype, animals lacking both α- and β2-syntrophins exhibit more pronounced defects at the NMJ than the α-syntrophin null (Adams et al., 2004). The number of junctional folds that open to the synaptic cleft is down to ~10% of wildtype numbers as opposed to the ~70% reduction compared to wildtype in α-syntrophin null. There was also a further reduction in the level of AChR found at the NMJ, at ~23% of the levels found at the wildtype synapse.

Taken together, the above studies demonstrate that, while DGC is not critical for the clustering of AChR *per se* at the developing NMJ, its members are crucial for maturation and/or stability of the synaptic receptor clusters at the myoneural junction.

Adenomatous polyposis coli (APC)

Adenomatous polyposis coli was originally identified as a tumor suppressor protein that is mutated in most colon cancers (van Es et al., 2001; Nathke, 2004). Subsequent studies demonstrated that APC acts primarily as a negative regulator of the canonical Wnt signaling pathway, and is expressed in a wide array of tissues, including

the nervous system. Interestingly, recent findings implicate APC in formation of cholinergic synapses (Wang et al., 2003; Temburni et al., 2004). Fluorescent microscopy data illustrated that APC is found at cholinergic synapses of chick ciliary ganglia (Temburni et al., 2004). In addition, the study showed that overexpression of dominant-inhibiting APC resulted in reduction in α3-AChR surface levels and clusters. Independently, APC was identified as an AChR interacting protein in muscle using the yeast two-hybrid system (Wang et al., 2003). Agrin-treatment of cultured myotubes increases AChR-APC interaction and induces clustering of APC. Overexpression of dominant-negative APC fragment also inhibits agrin-induced clustering of AChR.

It is not yet clear how APC is involved in postsynaptic clustering of AChR in muscle. One possibility is that APC serves as a molecular linker between the AChR and the cytoskeleton since it is able to interact with microtubules through its C-terminal basic domain (van Es et al., 2001). An alternative mode of APC action could be reorganization of actin cytoskeleton. APC binds to Asef, a guanine nucleotide exchange factor for Rho family of small GTPases, and potentiates its ability to activate Rac (Kawasaki et al., 2000). Consistent with this, agrin-treatment of myotubes in culture results in Rac activation, while dominant-negative Rac mutant expression prevented agrin-induced AChR clustering (Weston et al., 2000). The finding that APC may be required for AChR clustering also presents the possibility of a cross-talk between the agrin- and the canonical Wnt-signaling pathways. Consistent with this idea, a recent study has demonstrated that inhibitors of glycogen synthase kinase-3β inhibited agrin-induced AChR clustering and also increased the level of soluble β-catenin (Sharma and Wallace, 2003).

Regulation of AChR localization

Agrin signaling

Binding of Z+ agrin to the MuSK receptor complex activates the kinase domain of MuSK and initiates/stabilizes post-synaptic differentiation (Fig 4). Despite the identification of central players such as agrin, MuSK and rapsyn, the signaling pathway that leads to the assembly of the mature post-synaptic apparatus is still poorly understood.

Agrin signaling likely modulates interaction of postsynaptic proteins to synaptically cluster the AChR as the onset of expression for most the postsynaptic components precede innervation (Lin et al., 2001; Yang et al., 2001) and clustering of AChR occurs rapidly (<120 min) after nerve-muscle contact *in vitro* (Cohen and Godfrey, 1992; Cohen et al., 1995). Indeed, a major component of the agrin signaling cascade appears to be protein phosphorylation of postsynaptic components, a common post-translational modification and an efficient way to regulate protein-protein interactions. In particular, agrin signaling events appear to require activities of tyrosine kinases. First, MuSK is a receptor tyrosine kinase (Valenzuela et al., 1995; Glass et al., 1996), and at least one additional cellular tyrosine kinase is required downstream of MuSK to produce AChR clustering (Fuhrer et al., 1997). Second, inhibitors of tyrosine kinases inhibit agrin-induced AChR clustering in cultured myotubes (Wallace, 1994; Ferns et al., 1996), while a potent serine kinase inhibitor did not inhibit agrin-induced receptor clustering (Wallace et al., 1991). Third, phosphotyrosine immunoreactivity is associated with the NMJ *in vivo* (Qu et al., 1990), and nerve- or agrin-induced AChR clusters in culture (Wallace et al., 1991; Qu and Huganir, 1994). Forth, several postsynaptic proteins are tyrosine phosphorylated (Wagner et al., 1993; Balasubramanian and Huganir, 1999; Mittaud et al., 2001; Finn et al., 2003). And lastly, agrin induces phosphorylation and/or

activation of several enzymes, including several non-receptor tyrosine kinases (Mittaud et al., 2001; Finn et al., 2003), p21-Activated Kinase (PAK1) (Luo et al., 2002), and geranylgeranyltransferase-1 (Luo et al., 2003), which are critical for formation or stability of AChR clusters in muscle.

Tyrosine phosphorylation of AChR

During the initial phase of NMJ formation, redistribution of pre-existing surface AChR likely accounts for a large fraction of the receptor found at the synapse. Prior to clustering, AChR is free to diffuse within the plane of the plasma membrane and able to rotate about its central axis (Velez et al., 1990; Meier et al., 1995). Once clustered, the mobility of the receptor is reduced (Meier et al., 1995) and there is very little diffusion away from the NMJ (Akaaboune et al., 1999). These findings are in agreement with the "diffusion trap" model proposed by Edwards and Frisch (Edwards and Frisch, 1976), which states that a "sticky zone" is created within the muscle membrane where it is contacted by nerve where mobile AChR becomes trapped.

Several lines of evidence suggest that agrin-induced tyrosine phosphorylation of the AChR is an important step in the signaling pathway that leads to the formation of the post-synaptic AChR aggregates (Fig4). First, several studies have shown that agrin-induced phosphorylation of the β and δ subunit occurs prior to clustering of the AChR (Wallace et al., 1991; Qu and Huganir, 1994; Ferns et al., 1996; Mittaud et al., 2001; Mohamed et al., 2001). Next, tyrosine phosphorylation of the β and δ subunits, and clustering of the AChR are correlated under several experimental conditions and show identical dose-dependence to Z+ agrin (Ferns et al., 1996; Mittaud et al., 2001). Thirdly,

inhibitors of protein tyrosine kinases, such as herbimycin A and staurosporine that prevent the formation of new AChR clusters and disrupt pre-existing aggregates, also inhibit phosphorylation of the AChR β and δ subunits (Wallace, 1994; Ferns et al., 1996; Fuhrer et al., 1997; Mittaud et al., 2001; Mohamed et al., 2001). Fourth, increased tyrosine phosphorylation of AChR have been associated with reduced receptor mobility and extractability, possibly due to a stronger interaction between the AChR and the cytoskeletal network (Meier et al., 1995; Wallace, 1995). The above studies suggest that agrin-induced phosphorylation of AChR, and its subsequent interaction with the cytoskeleton, contributes to AChR clustering. Indeed, AChRs that lack β subunit phosphorylation clustered less efficiently, and agrin treatment did not increase cytoskeletal anchorage of mutant receptors (Borges and Ferns, 2001).

AChR lacking β subunit phosphorylation still clustered in response to agrin (Meyer and Wallace, 1998; Borges and Ferns, 2001). This finding, together with the fact that agrin-induced phosphorylation and cytoskeletal anchorage of AChR reaches maximum several hours before receptor clustering (Ferns et al., 1996; Borges and Ferns, 2001), raise the possibility that anchoring and clustering of the receptor are closely correlated, but nevertheless distinct processes that occur downstream of agrin signaling. Indeed, use of pharmacological agents demonstrated that the two processes could be uncoupled in cultured myotubes (Wallace, 1995; Sharma and Wallace, 2003). Regardless, agrin-induced β subunit tyrosine phosphorylation and the attachment of the AChR to the post-synaptic cytoskeletal network are likely to be intimately involved with the localization of the AChR at the mammalian NMJ.

Experiments with the electric organ of *Torpedo californica* have revealed that the β subunit of the AChR is phosphorylated by a tyrosine-specific protein kinase(s) (Huganir et al., 1984). MuSK does not seem to be directly responsible for the tyrosine phosphorylation of the β subunit of AChR since tyrosine phosphorylation (activation) of MuSK and that of the β subunit can be dissociated (Fuhrer et al., 1997). The identity of this protein tyrosine kinase(s) that is responsible for β subunit phosphorylation still needs to be determined. Src-class kinases have been shown to be in a stable complex with the AChR: Fyn and Fyk in *Torpedo* electric organ (Swope and Huganir, 1993), Src and Fyn in mammalian muscle (Fuhrer and Hall, 1996; Fuhrer et al., 1999). Src-class kinases have also been shown to phosphorylate the intracellular loop of the β subunit both *in vitro* (Fuhrer and Hall, 1996) and in a heterologous expression system (Mohamed and Swope, 1999). In addition, the AChR-bound pool of Src-class kinases is activated in response to the application of recombinant Z+ agrin (Mittaud et al., 2001). These findings suggest that Src-class kinases are responsible for the agrin-induced phosphorylation of receptor β subunit. Still, normal agrin-induced phosphorylation of the AChR β subunit occurs in myotubes null for src (src -/-), fyn (fyn -/-), or both (src -/-;fyn -/-) (Smith et al., 2001). Although a compensatory function of remaining Src-class kinases cannot be ruled out, this finding raises the possibility that kinases other than those belonging to the src-family may be responsible for agrin-induced AChR β subunit phosphorylation. Subsequently, a recent study has implicated Abl kinases (Abl-1 and Abl-2) in agrin-induced AChR clustering (Finn et al., 2003). First, Abl kinases were found concentrated postsynaptically at the NMJ *in vivo*. Next, agrin induced an increase in Abl-1 activity and also enhanced Abl-1's interaction with MuSK. Finally, a specific inhibitor of Abl kinases or expression

of Abl-1 with an inactive kinase domain effectively reduced the number of agrin-induced AChR clusters on cultured myotubes. It is, however, unclear whether the reduction in the number of receptor clusters was due to diminished β subunit phosphorylation, although a recent study suggests that Abl kinases might partially account for the agrin-induced β subunit phosphorylation (Mittaud et al., 2004). Also, while the findings suggest that Abl kinases are required for the agrin-induced AChR clustering, the knockout mutants of the Abl-1 and Abl-2 have functional NMJs and survive postnatally (Burden et al., 2003). The Abl-1 and -2 double knockout, unfortunately, die early in embryogenesis before NMJs begin to form (Burden et al., 2003). This suggests that the two Abl kinases have redundant functions and one may compensate for the other, but it is currently not clear whether Abl kinases are critical for development of the NMJ. Alternatively, it is possible that multiple kinases, including but not limited to Src and Abl families of kinases, act co-operatively in order to produce AChR clustering in response to agrin (Mittaud et al., 2004). Therefore, despite the intensive efforts to date, further studies are required to identify kinase(s) responsible for mediating AChR phosphorylation.

Interestingly, agrin-induced AChR β subunit phosphorylation is significantly reduced in rapsyn null myotubes (Apel et al., 1997; Mittaud et al., 2001). The virtual lack of β subunit phosphorylation despite the activation of MuSK seen in rapsyn null myotubes suggests that rapsyn is required for the agrin-induced signaling cascade. This, in turn, indicates that, in addition to serving as a physical link between the MuSK-containing primary scaffold and components of the post-synaptic apparatus, rapsyn is an active player in the agrin signaling pathway that result in tyrosine phosphorylation of the AChR. Co-immunoprecipitation data suggest that rapsyn interacts with Src-family

kinases, and AChR phosphorylation mediated by Src-family kinases is potentiated several fold by rapsyn co-expression (Mohamed and Swope, 1999). Rapsyn-dependent activation of Src-family kinases with agrin treatment further supports this view (Mittaud et al., 2001). Therefore, rapsyn may perform this signaling role by regulating the activity and/or through recruitment of protein tyrosine kinases and other molecules that are critical for AChR phosphorylation.

Synaptic transcription of AChR subunit genes

Enrichment of AChR mRNA at the endplate region of the muscle (Merlie and Sanes, 1985; Fontaine et al., 1988; Goldman and Staple, 1989) is a second major contributor to the dense accumulation of the receptor at the synapse. This is done in two ways: preferential transcription of AChR subunit genes from the synaptic nuclei of the muscle (Sanes et al., 1991; Simon et al., 1992) and downregulation of the same genes from extrasynaptic nuclei by nerve-evoked electrical activity of the muscle (Lomo and Rosenthal, 1972; Berg and Hall, 1975; Merlie et al., 1984; Fontaine et al., 1988; Goldman et al., 1988; Goldman and Staple, 1989; Tsay and Schmidt, 1989). Concentration of transcripts for the AChR subunits and other synaptic proteins allows the muscle to locally synthesize and utilize proteins precisely where they are needed.

Interestingly, several findings suggest that agrin-MuSK signaling also contributes to synapse-specific transcription of AChR subunit genes. Ectopic expression of Z+ agrin in innervated muscle resulted in localized increased transcription of AChR ε subunit gene, in addition to ectopic AChR clustering (Jones et al., 1997; Meier et al., 1998). Moreover, there is a diffuse distribution of AChR subunit transcripts along the length of the muscle fibers in Z+ agrin null mice, in contrast to the enrichment in the endplate

region of wildtype muscle (Gautam et al., 1996). More recent genetic experiments show that synapse-specific expression of AChR does not require innervation (Lin et al., 2001; Yang et al., 2001), and therefore Z+ agrin, but requires MuSK expression (Yang et al., 2001). Therefore, while establishment of transcriptionally specialized zone is MuSK-dependent and agrin-independent, the maintenance of such specialization requires nerve-derived agrin to overcome the effects of nerve-evoked muscle activity as innervation procedes.

Interestingly, constitutively active MuSK did not directly increase transcription of AChR ε subunit in cultured myotubes (Jones et al., 1999). This suggests that while agrin-MuSK signaling is required, it is not sufficient and requires intermediary factors and/or signaling pathways to upregulate AChR expression. An additional nerve-derived factor, neuregulin-1 (initially named ARIA for acetylcholine receptor-inducing activity), was identified from chick brain extract based on its ability to enhance expression of AChR subunit genes in culture (Harris et al., 1988; Martinou et al., 1991; Falls et al., 1993; Chu et al., 1995). In addition, neuregulin-1 and its receptors, ErbBs, are concentrated at the NMJ (Chu et al., 1995; Goodearl et al., 1995; Jo et al., 1995; Moscoso et al., 1995b; Zhu et al., 1995). Moreover, neuregulin is produced by both motoneurons and skeletal muscle fibers (Moscoso et al., 1995b; Meier et al., 1998), and ectopic expression of MuSK is sufficient to induce local expression of ErB-2 and -3 (Moore et al., 2001). Therefore, it is possible that muscle-derived neuregulin is responsible for transcriptional pattering of muscle fibers. Surprisingly, a recent genetic study showed that motor neuron- and skeletal muscle-derived neuregulin-1 is dispensable for high-density accumulation of the AChR in the postsynaptic muscle membrane as well as for the synapse-specific transcription of AChR subunit genes (Jaworski and Burden, 2006). Neuregulin-2,

expressed by motor neurons and terminal Schwann cells, enhances AChR transcription in cultured muscle cells (Rimer et al., 2004), raising the possibility that it may compensate for the loss of neuregulin-1 expression. However, synapse-specific accumulation of AChR and its mRNA occurred normally in absence of neuregulin signaling receptors, ErbB2 and ErbB4 (Escher et al., 2005), indicating that neuregulin-signaling in muscle is dispensable for postsynaptic differentiation, and, furthermore, that its contribution to NMJ formation may be secondary to promoting survival of terminal Schwann cells (Jaworski and Burden, 2006). Therefore, while agrin-MuSK signaling contributes to synapse specific expression of AChR, it is unlikely through clustering of neuregulin or erbB receptors as initially suspected.

Regulation of AChR metabolic stability

Another mechanism that contributes to the synaptic localization of AChR is the developmental change in its metabolic stability. The progressive development of the post-synaptic apparatus is associated with a change in the AChR turnover rate at the mammalian NMJ, from approximately one day to more than 10 days (Hall and Sanes, 1993; Sanes and Lichtman, 1999). The precise molecular/cellular events responsible for the change in the turnover-time of AChR is unclear, however this phenomenon appears to be influenced by not a single, but several processes including muscle activity, anchorage of the AChR to the cytoskeleton, and even subunit composition of the AChR (Salpeter, 1999).

The post-natal switch in the composition of the AChR (from fetal γ subunit to adult ε subunit) has been implicated in metabolic stabilization of the synaptic AChR as

the ε subunit containing AChR belong to the pool of the receptor with slower turnover rate (Sala et al., 1997). However, turnover rate of the ε subunit containing "adult" AChR can be modulated post-translationally (Sala et al., 1997). Therefore, the subunit composition of AChR cannot be entirely responsible for the nearly 10-fold decrease in the rate of receptor turnover, and additional cellular processes must also be involved in regulating receptor stability at the synapse.

Several studies indicate that muscle activity may also contribute to the regulation of AChR stability at the synapse. Pharmacological blockade of muscle activity *in vivo* reverses the increase in AChR half-life, whereas it can be mimicked by direct electrical stimulation of muscle (Akaaboune et al., 1999). Presently, it is unclear how muscle activity might slow the degradation of synaptic AChR. It is has been suggested that anchorage of AChR to the cytoskeleton could contribute to the increase in its metabolic stability (Salpeter, 1999). Recent reports demonstrated that agrin has a positive effect on the metabolic stability of the AChR *in vivo* (Bezakova et al., 2001), and that agrin-induced phosphorylation of AChR β subunit regulates receptor anchorage to the cytoskeleton (Borges and Ferns, 2001). Consistent with this, another recent study suggests that increase in AChR β subunit phosphorylation is associated with AChR stabilization in culture (Sava et al., 2001).

As rapsyn aggregates and anchors AChR in heterologous cells, it is believed to be the protein that links the AChR to the cytoskeleton at the postsynaptic apparatus. Rapsyn, therefore, may help decrease the rate of AChR turnover at the NMJ. Therefore, the AChR-rapsyn interaction, possibly subject to regulation by agrin-induced AChR β subunit phosphorylation, may help stabilize AChR by increasing its linkage to the

underlying cytoskeleton (Borges and Ferns, 2001). Indeed, studies utilizing heterologous expression systems support a role for the rapsyn-AChR interaction in regulation of AChR stability (Phillips et al., 1997; Wang et al., 1999). However, while rapsyn seems to participate in the metabolic stabilization of the AChR in muscle, there is a controversy in the literature as to the mechanism of its action (Phillips et al., 1997; Wang et al., 1999). The AChR-rapsyn interaction, possibly subject to regulation by agrin-induced AChR β subunit phosphorylation, may help stabilize synaptic AChR clusters by increasing its linkage to the underlying cytoskeleton (Borges and Ferns, 2001).

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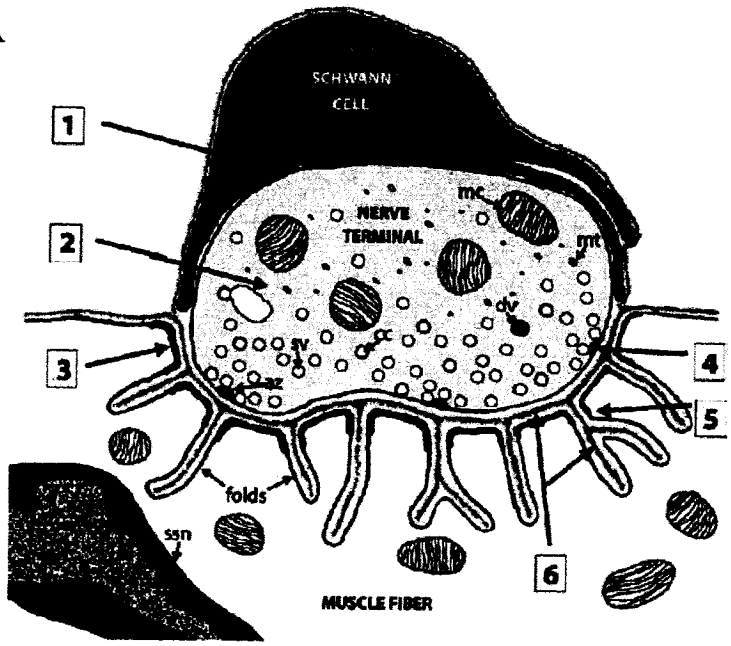
Figure 1. Structure and molecular composition of the neuromuscular junction

(A) The vertebrate neuromuscular junction (NMJ) contains three cellular components: [1] the terminal Schwann cells which caps the junction, [2] the presynaptic motor nerve terminal bearing a large number of synaptic vesicles, and [3] the AChR-rich postsynaptic muscle membrane. The active zones [4] are precisely aligned with the openings of the synaptic folds [5] to ensure efficient synaptic transmission. A specialized form of the muscle basal lamina [6] is found in the synaptic cleft. az, active zone; mc, mitochondria; sv, synaptic vesicle. (Modified from Patton, 2003) **(B)** Subcellular distribution of synaptic molecules. (Modified from Sanes and Lichtman, 1999)

A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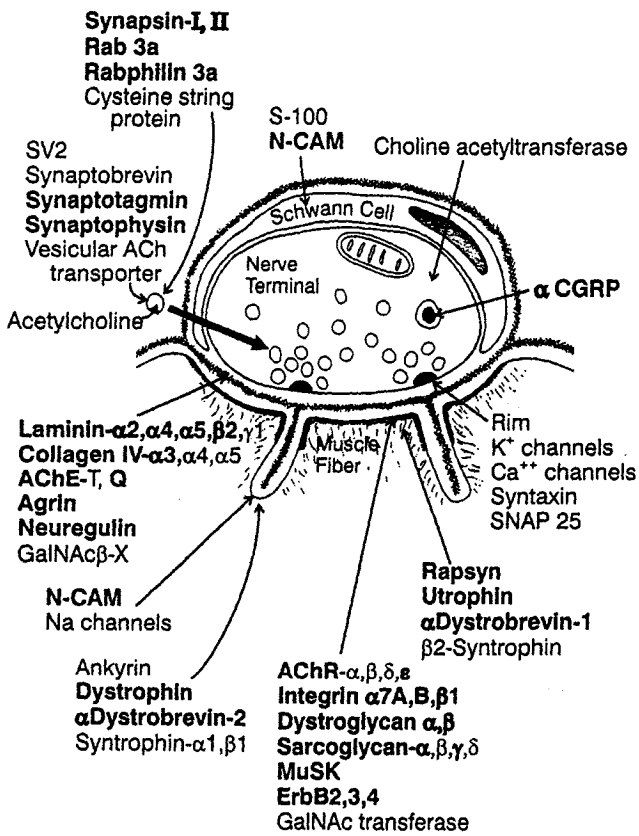
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Synaptobrevin
Synaptotagmin
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S-100
N-CAM
Choline acetyltransferase
Schwann Cell
Nerve Terminal
α CGRP
Laminin-α2,α4,α5,β2,γ1
Collagen IV-α3,α4,α5
AChE-T, Q
Agrin
Neuregulin
GalNAcβ-X
Muscle Fiber
Rim
K+ channels
Ca++ channels
Syntaxin
SNAP 25
N-CAM
Na channels
Ankyrin
Dystrophin
αDystrobrevin-2
Syntrophin-α1,β1
AChR-α,β,δ,ε
Integrin α7A,B,β1
Dystroglycan α,β
Sarcoglycan-α,β,γ,δ
MuSK
ErbB2,3,4
GalNAc transferase
Rapsyn
Utrophin
αDystrobrevin-1
β2-Syntrophin

Figure 2. Development of the vertebrate neuromuscular junction

(A) Numerous observations support the "neurocentric" view of neuromuscular synapse formation, in which the motor nerve directs the formation and location of AChR clusters on the muscle fiber. (B) Recent genetic studies demonstrated that muscle is pre-patterned prior to innervation, and form autonomous AChR clusters which can be recognized by innervating motor axons and incorporated into synapses.

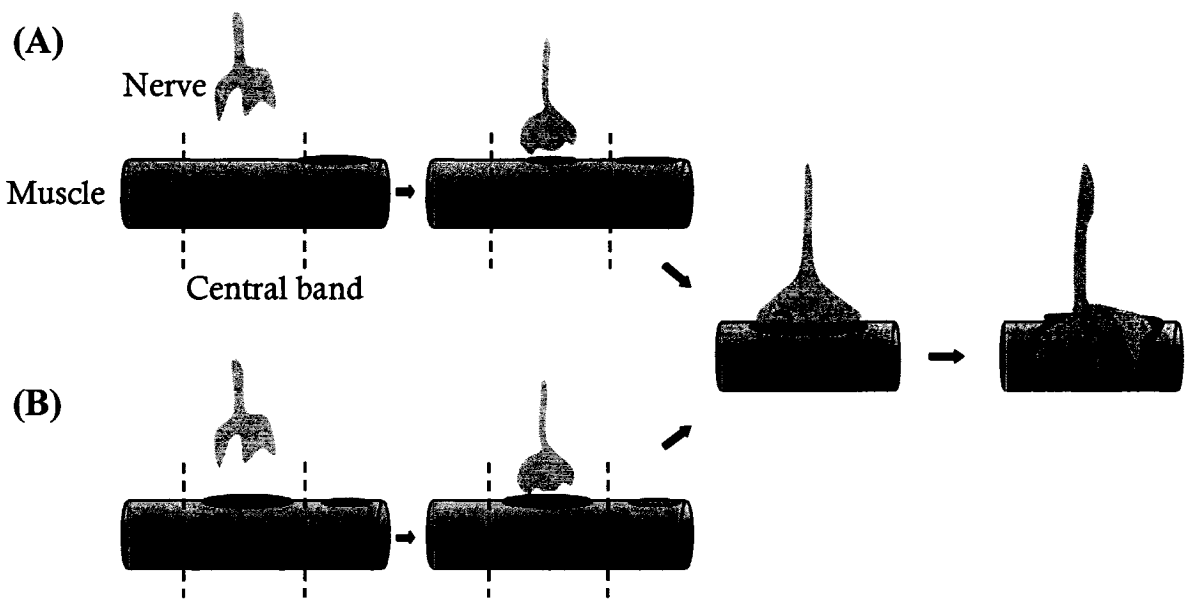

(A)
Nerve
Muscle
Central band
(B)

Figure 3. Rapsyn structure

Schematic showing the domain structure of rapsyn based on analysis of its primary amino-acid sequence. The N-terminal myristoylation targets rapsyn to the cell membrane. The seven tetratricopeptide repeats (TPRs) mediate rapsyn self-association. The C-terminal coiled-coil and RING domains mediate rapsyn interaction with AChR and β-dystroglycan, respectively.

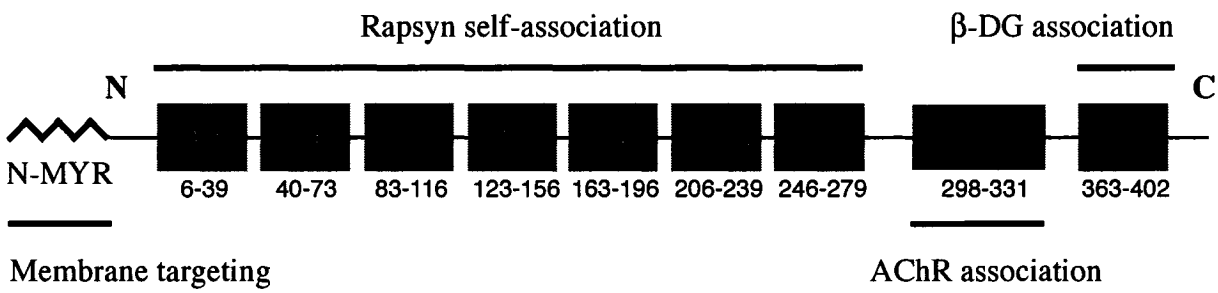
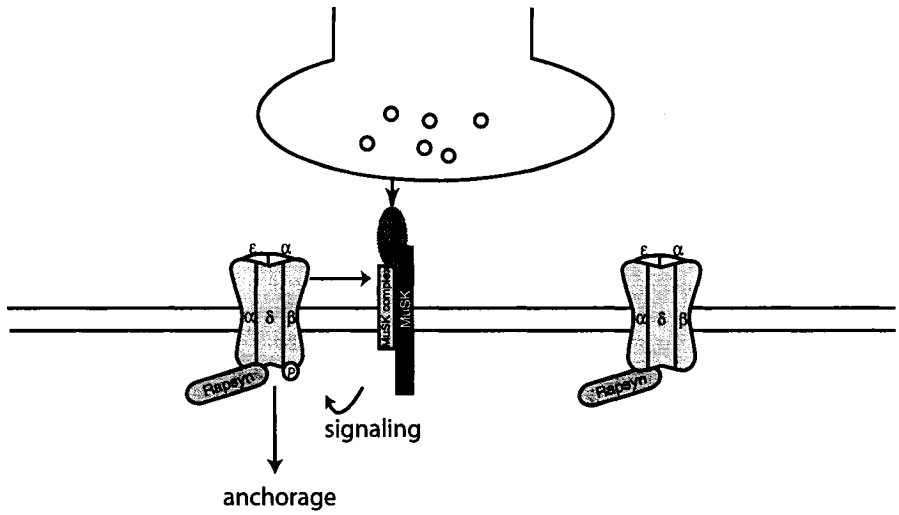

Rapsyn self-association
β-DG association
N
C
N-MYR
6-39
40-73
83-116
123-156
163-196
206-239
246-279
298-331
363-402
Membrane targeting
AChR association

Figure 4. Model for agrin signaling and synaptic AChR localization

(A) Motoneuron-derived Z+ agrin activates MuSK and initiates a local signaling cascade that induces tyrosine phosphorylation of AChR β subunit. Phosphorylated AChR becomes progressively clustered and anchored at the neuromuscular junction. (B) Cytoskeletal anchorage of the receptor could occur directly via its interaction with rapsyn, or indirectly through rapsyn's association with dytrophin/utrophin-glycoprotein complex through β-dystroglycan.

A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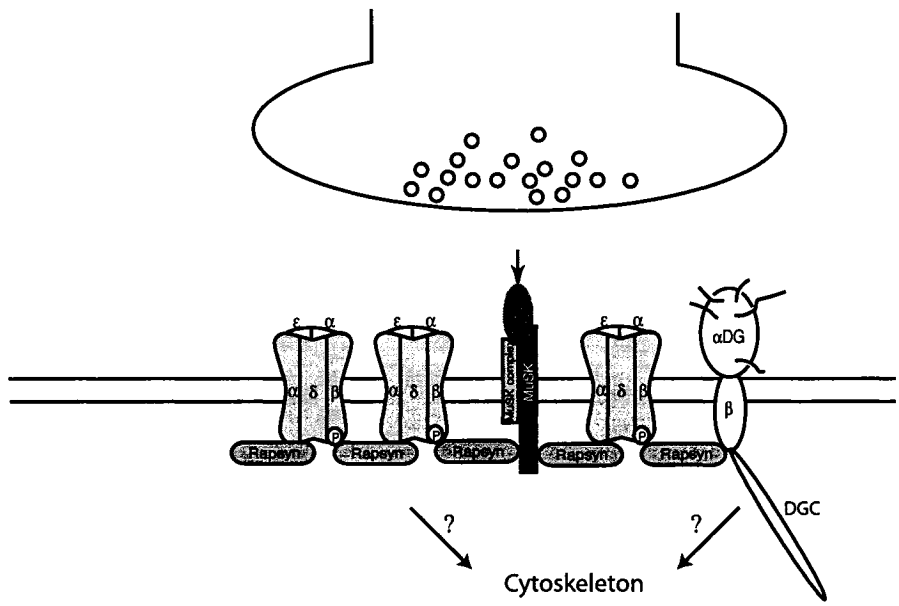
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Cytoskeleton

Chapter II

Rapsyn interacts with the AChR via an α-helical motif conserved between the α, β, and ε subunit intracellular loop

Young Lee and Michael Ferns

In preparation

ABSTRACT

At the developing vertebrate neuromuscular junction, the AChR becomes aggregated at high density in the postsynaptic muscle membrane. Receptor localization is regulated by the motoneuron-derived factor, agrin, and requires an intracellular, scaffolding protein called rapsyn. However, it is unclear where rapsyn binds on the AChR and if their interaction is constitutive or regulated. In this study, we identified rapsyn's binding site on the AChR using chimeric constructs where the intracellular domain of CD4 was substituted for the major intracellular loop of each AChR subunit. When expressed in heterologous cells, we found that rapsyn clusters and cytoskeletally anchors CD4- α, β and ε subunit loops, although with differing efficiencies. Moreover, we show that rapsyn interacts with the α-helical region, a secondary structural motif in the C-terminal portion of the loops. In muscle cells, we also found that rapsyn co-immunoprecipitated together with a CD4- α helical region chimera, independent of agrin-signaling. Together, these findings demonstrate that rapsyn interacts with the AChR by binding an α-helical structural motif conserved between the α, β and ε subunits. This provides 4 potential binding sites per receptor, potentially allowing for both constitutive and regulated rapsyn interactions involved in synaptic localization of the AChR.

INTRODUCTION

At the developing neuromuscular junction, presynaptic nerve terminals become precisely aligned with high-density aggregates of AChR in the postsynaptic membrane, ensuring the high fidelity of transmission at this synapse. This process is regulated in part by agrin, a motoneuron-derived factor that plays an essential role in stably aggregating the AChR at nascent synapses (Sanes and Lichtman, 1999; Kummer et al., 2006). Agrin induces and/or stabilizes AChR clusters by activating the muscle-specific kinase (MuSK), and nerve-associated receptor clusters are absent in agrin or MuSK null mice at birth, resulting in perinatal lethality (DeChiara et al., 1996; Gautam et al., 1996). MuSK activation induces several downstream signaling events, including activation of the kinase PAK1 (Luo et al., 2002), the small GTPases Rac, cdc42, and Rho (Weston et al., 2000; Weston et al., 2003), and the cytoplasmic tyrosine kinases src/fyn (Mittaud et al., 2001) and abl/arg (Finn et al., 2003), which may phosphorylate the AChR. However, the specific signaling events and protein interactions that mediate clustering of the AChR are still not clear (Strochlic et al., 2005).

One critical mediator of AChR localization is the intracellular, membrane-associated scaffolding protein, rapsyn. Rapsyn colocalizes precisely with AChR clusters *in vitro* and *in vivo* (Froehner et al., 1981; Burden, 1985; Noakes et al., 1993), and is estimated to be in approximately 1:1 stoichiometry with the AChR (Burden et al., 1983; LaRochelle and Froehner, 1986). Moreover, rapsyn clusters the AChR (and several other synaptic proteins) when they are co-expressed in heterologous cells (Froehner et al., 1990; Phillips et al., 1991b), and AChR clusters fail to form at neuromuscular contacts in rapsyn null mice (Gautam et al., 1995). In addition, rapsyn mutations in humans result in

decreased AChR levels at the synapse, producing a severe myasthenic syndrome with impaired transmission and debilitating muscle weakness (Ohno et al., 2002; Maselli et al., 2003). In fact, up to 10% of congenital myasthenic syndromes may be due to rapsyn mutations (Engel et al., 2003; Maselli et al., 2003).

Surprisingly, the molecular mechanism by which rapsyn localizes the AChR remains unknown. Functional studies on rapsyn have revealed an N-terminal myristylation site required for rapsyn's targeting to the plasma membrane (Phillips et al., 1991a), 7 tetratricopeptide repeats (aa 6-279) that mediate rapsyn self-association (Ramarao and Cohen, 1998; Ramarao et al., 2001), and a coiled-coil domain (aa 298-331) and cysteine-rich RING structure (aa 363-402) required for clustering of AChR and β-dystroglycan, respectively (Ramarao and Cohen, 1998; Bartoli et al., 2001; Ramarao et al., 2001). Rapsyn's site of interaction on the AChR is unclear, however, and it is unknown whether rapsyn binds one or more subunits of the pentameric AChR ($\alpha_2\beta\varepsilon\delta$ or $\alpha_2\beta\gamma\delta$) and if binding is constitutive or regulated. Indeed, each subunit contains a large intracellular loop between the third and fourth transmembrane domains that could potentially interact with rapsyn, and consistent with this, structural studies on the AChR have revealed an accessory protein, that is presumably rapsyn, lying immediately beneath the channel in association with the loops (Miyazawa et al., 1999). One candidate interaction site is the β subunit loop, as cross-linking studies have shown that rapsyn is in close proximity to this subunit (Burden et al., 1983). On the other hand, it has been reported that rapsyn can associate with all of the AChR subunits when individually expressed in heterologous cells, at least in intracellular aggregates (Maimone and Merlie, 1993; Huebsch and Maimone, 2003).

Here, we have mapped rapsyn's binding site on the AChR using CD4- subunit loop chimeric proteins. We find that rapsyn interacts selectively with the α, β and ε subunit loops in heterologous cells but with differing apparent affinities. Moreover, we show that rapsyn interacts with the α-helical region, a secondary structural motif in the C-terminal portion of the subunit loops. As rapsyn binding to this isolated region was not regulated by agrin in muscle cells, we propose that it forms the primary, constitutive binding site for rapsyn on the AChR.

MATERIALS AND METHODS

CD4-subunit loop constructs

To generate the CD4- subunit loop chimeras, a Bgl II restriction site was introduced by site directed mutagenesis at the end of the transmembrane domain of the mouse CD4 cDNA. The intracellular domain of CD4 was then excised and the large intracellular loops of the AChR subunits were ligated into this site. The loops were obtained by PCR of mouse cDNAs for each of the AChR subunits, and all the constructs were then sequenced. All the CD4/loop constructs were expressed in the mammalian expression vector pcDNA3.

To generate CD4-β loop α-helix constructs, a BglII site was introduced at the end of the CD4 intracellular domain and PCR fragments comprising segments of the α-helix were ligated into this site (denoted CD4ct-β). This CD4 tail spacer was used to position the short α-helical segment further from plasma membrane.

Cell culture and transfection

COS cells were grown in Dulbecco's modified eagle medium with high glucose (DMEM-HI), supplemented with 10% fetal bovine serum and 100U/ml penicillin-streptomycin. For biochemical experiments, cells growing in 10cm dishes were transfected using the $CaPO_4$ method. For immunostaining experiments, cells growing in eight well chamber slides (Nalge Nunc Itl. Naperville, IL) were transfected in parallel.

Sol8 mouse muscle cells were maintained in DMEM-HI, supplemented with 20% fetal bovine serum, 100U/ml of penicillin-streptomycin, and 2mM L-glutamine. The myoblasts were transfected at 85% confluency using the $CaPO_4$ method, and then when

confluent, the cells were incubated with fusion medium (DMEM-HI supplemented with 5% horse serum and 2mM L-glutamine) to induce formation of myotubes.

Protein extraction, immunoprecipitation and western blotting

To assay detergent extractability, transfected COS cells were rinsed, scraped off and pelleted in ice-cold PBS. They were then re-suspended in extraction buffer (25mM Tris, 25mM glycine, 150mM NaCl, 5mM EDTA, 1% Triton X-100 and the protease inhibitors PMSF, benzamidine and $Na_2S_4O_6$) and incubated for 10 minutes on ice, after which the insoluble proteins were pelleted by centrifugation (16,100 g for 4 min). The CD4 chimeric proteins were then immunoprecipitated from the soluble fraction with monoclonal antibody GK1.5 (Pharmigen) and protein G-agarose (Invitrogen). In addition, the insoluble pellet was resuspended in 200 µl of loading buffer (SDS, glycerol, 5% β-mercaptoethanol and bromophenol blue) and boiled. The immunoprecipitated proteins and 20% of the pellet fraction were separated on 10% polyacrylamide gels. They were then immunoblotted with monoclonal antibody H129.19 to CD4 (Pharmigen), followed by an anti-rat HRP-conjugated secondary antibody and visualized using enhanced chemiluminescence. The intensity of the western blot signals was quantified using Sci-Scan 5000 Bioanalysis (USB; Cleveland, OH) or ImageGauge V4.22 software (Fuji Film).

To assay rapsyn association in Sol8 myotubes, the cells were extracted and CD4 immunoprecipitations performed as above. The AChR was also isolated using biotin-conjugated α-bungarotoxin and streptavidin agarose (Molecular Probes). Co-immunoprecipitated rapsyn was then detected by immunobotting with polyclonal

antibody B5668 (generated against a peptide encompassing aa's 133-153) (Moransard et al., 2003). The blots were reprobed for the AChR α subunit using mAb210 (Covance).

Immunostaining of transfected cells

Transfected COS cells were fixed with 2% paraformaldehyde/PBS, blocked with 10% horse serum/PBS and stained for surface CD4 with rat monoclonal antibodies GK1.5 or H129.19 (Pharmigen). After washing for 10 minutes with PBS, the cells were permeabilized in 0.5% Triton X-100/PBS for 10 minutes, washed with PBS, and incubated with rapsyn monoclonal antibody 1234 for 45 minutes (gift of Dr. Froehner, U. Wash.). The cells were then incubated with Alexa 488-conjugated anti-mouse and Alexa 594-conjugated anti-rat antibodies (Molecular Probes).

To quantify rapsyn-induced clustering of CD4- subunit loops, we selected random fields and then scored all rapsyn-positive cells according to whether they had strong, weak or no clustering of CD4. Cells were defined as having strong clusters when CD4 staining overlapped precisely with rapsyn aggregates with little staining elsewhere, and weak clusters when significant CD4 staining was evident elsewhere on the cell surface.

RESULTS

Construction and expression of CD4-subunit loop chimeras

The muscle AChR is a pentamer composed of 2 α, β, δ and γ (fetal) or ε (adult) subunits. The subunits share ~31% homology (~16% sequence identity) and have the same membrane topology, with a large intracellular loop between transmembrane domains 3 and 4 that is the most likely site of interaction with rapsyn. To define rapsyn's binding site on the AChR, therefore, we generated chimeric constructs where the intracellular domain of CD4 was substituted for the major intracellular loop of each AChR subunit (Fig 1A,B). This allowed surface expression and circumvents the problem that individually expressed subunits are retained in the endoplasmic reticulum, with only fully assembled AChR being trafficked to the cell surface. Thus, it enabled us to test rapsyn's interaction with each subunit loop and map the binding domain.

To confirm expression of the CD4-subunit loop chimeras, we transiently transfected the constructs into COS cells and then immunoprecipitated and immunoblotted the chimeric proteins using an antibody to the CD4 extracellular domain. We found that all the CD4-loop chimeras were highly expressed and that the proteins ran at the expected molecular weights (Fig 1C). Reprobing with antibodies specific to the β (mAb124) and δ (mAb88b) intracellular loops also identified the respective CD4-subunit loop constructs.

Rapsyn selectively clusters CD4-α, -β and -ε subunit loops

To test whether rapsyn interacts with one or more AChR subunit, we first assayed rapsyn-induced clustering of each CD4-subunit loop in heterologous cells. To do this, we

expressed each CD4-subunit loop chimera in COS cells, either alone or together with rapsyn, and then immunostained specifically for surface CD4 loop chimeras and then for intracellular rapsyn. When expressed alone, we found that CD4 and all the CD4 chimeras were expressed on the cell surface in a diffuse pattern (Fig 2A). When co-expressed with rapsyn, however, we found that CD4-α, -β, and -ε chimeras formed surface clusters that colocalized with rapsyn aggregates, whereas CD4 and CD4-δ remained diffusely distributed (Fig 2A). To compare the efficiency of rapsyn-induced clustering of the different subunit loop chimeras, we quantified the percentage of rapsyn-expressing cells that had clusters of CD4 loop chimeras and classified the clustering as weak or strong (Fig 2B; see Materials and Methods). We found that rapsyn induced little clustering of CD4 and CD4-δ, with these proteins being diffusely distributed in the majority of the cells (~80%). In contrast, rapsyn induced strong clustering of CD4-β and ε loops in most cells (70-84%) (Fig 2B), and clustering of CD4-α loop occurred at intermediate levels. Thus, we find that rapsyn clusters a subset of the AChR subunit loops (α,β,ε) and with differing efficiencies. Moreover, individual subunit loops are sufficient for rapsyn interaction.

Rapsyn anchors CD4-α, -β and -ε loops

Rapsyn was previously shown to anchor the AChR to the cytoskeleton in heterologous cells (Phillips et al., 1993; Mohamed and Swope, 1999). Therefore, we tested whether rapsyn also induced anchoring of the CD4 subunit loop chimeras, assayed by monitoring their detergent extractability as described previously (Kassner et al., 1998; Mohamed and Swope, 1999). Briefly, transfected cells were extracted with buffer

containing 1% Triton X-100 for 10 minutes on ice, and the distribution of the chimeras in the soluble versus insoluble (cytoskeleton-containing pellet) fraction was determined by immunoblotting. When expressed alone, we found that CD4 and all the CD4-subunit loop chimeras were almost completely solubilized in 1% Triton X-100 buffer (Fig 3A,B). When co-expressed with rapsyn, however, we found that the detergent extractability of CD4-α, -β, and -ε loops were significantly reduced and they were now retained to varying degrees in the cytoskeletal fraction. Notably, rapsyn induced anchorage of ~90% of CD4-β loop, 60% of CD4-ε loop and 50% of CD4-α loop protein (Fig 3B). These results confirm that rapsyn interacts selectively with 3 subunit loops and with different apparent affinities (β > ε > α subunit loop).

Interestingly, in these experiments, we also observed that the total level of CD4-β loop protein was increased ~9 fold in the presence of rapsyn (Fig 3C); this most likely stems from decreased turnover (Phillips et al., 1997; Wang et al., 1999) associated with the strong rapsyn-induced clustering and anchoring of the β loop but this remains to be confirmed.

Rapsyn interacts with α-helix region of β loop

Next, we defined the region of the intracellular loop that interacts with rapsyn by assaying additional CD4-loop deletion constructs, focusing on the β subunit as it displayed the strongest interaction with rapsyn. First, we tested two loop fragments; CD4-βE9, which contains the first half of the β-loop encoded primarily by exon 9 (E9; aa 333-406), and CD4-βE10, which contains the second half of the β-loop encoded by exon 10 (E10; aa 407-469). When co-expressed in COS cells, we found that rapsyn induced

robust clustering of CD4-βE10 but not CD4-βE9, which remained evenly distributed on the cell surface (Fig 4A). Similarly, rapsyn reduced the detergent extractability of CD4-βE10, with ~40% of the chimeric protein becoming cytoskeletally-anchored, but had no effect on the detergent extractability of CD4-βE9 (Fig 4B,C). Thus, rapsyn binds a region in the second half of the loop.

Although the AChR subunit loops share little sequence homology (~5% identity and ~14% similarity), they all contain predicted α-helical structures in the C-terminal loop region analogous to βE10 (Fig 1B). The precise position and length of the helices is unclear, with different studies (Finer-Moore and Stroud, 1984; Le Novere et al., 1999) or secondary-structure prediction programs (SSThread; (Ito et al., 1997)) giving variable results; however, α-helices are apparent in each of the subunit loops in a 4Å resolution structure derived from electron microscopy of purified Torpedo AChR-rich membrane (Unwin, 2005). We tested, therefore, whether the β loop α-helix was sufficient for rapsyn interaction and whether a specific region or sequence within the helix was involved (~ aa431-469; Fig 1B). Interestingly, we found that rapsyn clustered both $CD4^{ct}$-β431-449 and $CD4^{ct}$-β450-469 when co-expressed in COS cells (data not shown). Rapsyn also cytoskeletally anchored both regions of the helix, with ~30% of $CD4^{ct}$-β431-449 and ~55% of $CD4^{ct}$-β450-469 being retained in the detergent-insoluble fraction (Fig 5A,B). The anchoring of the first and second halves of the helix was less efficient than the rapsyn-induced anchoring of $CD4^{CT}$-βE10 (~80%), however.

Finally, we tested whether rapsyn interacts with $CD4^{ct}$-βE10 in muscle and if agrin signaling regulates their binding. To do this, we expressed $CD4^{ct}$-βE10 in Sol8 myotubes by transient transfection, either alone or together with additional rapsyn, and

treated with agrin (C-Ag4,8; 100 pM, 1 hr); we then immunoprecipitated $CD4^{ct}$-βE10 and the AChR from cell extracts and immunoblotted for rapsyn. Rapsyn was readily detected in association with the AChR, but only trace amounts co-immunoprecipitated together with $CD4^{ct}$-βE10 when expressed alone. This suggests that most rapsyn may be bound to the endogenous AChR, with little free pool available for binding of the introduced construct. Consistent with this, we found that rapsyn was co-immunoprecipitated with $CD4^{ct}$-βE10 when they were co-transfected into the muscle cells. This was not mediated indirectly via the AChR, as no association was observed between $CD4^{ct}$-βE10 and the receptor (Fig 6A). Moreover, no rapsyn association was observed with control constructs such as CD4 (Fig 6B). Interestingly, agrin treatment increased rapsyn association with the endogenous AChR but had no effect on rapsyn interaction with $CD4^{ct}$-βE10. Thus, agrin does not regulate rapsyn interaction with the isolated βE10 region.

DISCUSSION

The localization of the AChR at the developing neuromuscular junction is mediated by the scaffolding protein rapsyn but their mode of interaction is poorly understood. Here, we have mapped rapsyn's binding site on the AChR using CD4-subunit loop chimeric proteins and find that rapsyn selectively clusters and anchors the major intracellular loop of the α, β, and ε subunits. This interaction occurred efficiently in heterologous cells and was also evident in co-immunoprecipitations from muscle; thus, it most likely reflects direct binding of rapsyn to the subunit loop. Moreover, as the isolated loops of each subunit were sufficient for rapsyn interaction, this suggests that there are up to 4 independent rapsyn binding sites on the assembled AChR (ie. 2α/ 1β/ 1ε loop). The 4 sites are not equivalent, however, as we observed consistent differences in the relative levels of interaction of rapsyn with the three subunit loops, with rapsyn-mediated clustering and anchoring being highest for β subunit loop, followed by ε and then α. The simplest interpretation of these findings is that rapsyn binds the loops with differing affinities, preferentially interacting with the β and ε subunit loops. These findings are consistent with cross-linking studies showing rapsyn association with the β (Burden et al., 1983) and perhaps γ subunits (fetally-expressed homolog of the ε subunit) (Shoji et al., 1992). Previous studies have also shown that the stoichiometry of rapsyn to AChR in synaptic membranes is in the range of 0.5-2 (LaRochelle and Froehner, 1986), and a 4Å structure derived from electron images of tubular Torpedo membranes shows a two-fold symmetry for rapsyn, implying that 2 rapsyn molecules associate with a single AChR (Mitra et al., 1989; Miyazawa et al., 1999). Thus, only 1-2 of the 4 potential rapsyn-binding sites are likely utilized in a given AChR.

Our finding that rapsyn can interact with multiple loops suggests that they share a common binding motif, however little homology is evident in the primary sequences of the 3 interacting loops. Rather, we find that rapsyn interacts with the α-helix region of β loop, a predicted secondary structure conserved between the subunits. Indeed, α-helices of approximately 40 aa each are predicted in the C-terminal portion of the α, β and ε subunits (Le Novere et al., 1999). One notable exception is the delta subunit loop that is predicted to contain a shorter helix (SSThread; (Ito et al., 1997)), and intriguingly, we fail to detect rapsyn interaction with this subunit. Rapsyn binding to the α-helix presumably involves its coiled domain (Ramarao and Cohen, 1998; Ramarao et al., 2001) and likely involves recognition of secondary structure of the helix rather than primary sequence given the relatively low similarity between the subunit loops. Moreover, while we detected weak rapsyn-induced clustering and anchoring of the first or second halves of the α-helix, our experiments consistently show rapsyn binds with highest affinity to the complete α-helix. Interestingly, an α-helical region has also been implicated in the synaptic localization of the glycine receptor, forming the binding site for gephyrin on the glycine receptor beta2 subunit intracellular loop (Meyer et al., 1995; Kneussel et al., 1999). Our findings suggest, therefore, that this structural motif may be involved in the localization of more than one type of ion channel.

Rapsyn-mediated clustering of the AChR is regulated by agrin signaling in muscle. Although the molecular mechanism is unclear, it seems to involve both constitutive and regulated interactions between rapsyn and the AChR. A constitutive interaction may occur prior to clustering as rapsyn associates with the AChR in the late secretory pathway and is co-transported to the cell surface (Bignami et al., 1998;

Marchand et al., 2000; Marchand et al., 2002). Moreover, rapsyn co-immunoprecipitates with unclustered AChR in denervated muscle or cultured myotubes (Moransard et al., 2003), and is downregulated following antibody-induced internalization and degradation of surface AChR (Marangi et al., 2001). A regulated interaction may be required for clustering, however, as both preformed and nerve-induced AChR clusters fail to form in MuSK knockout muscle (DeChiara et al., 1996; Lin et al., 2001) and agrin treatment of cultured myotubes increases the amount of rapsyn co-immunoprecipitated with the AChR (Moransard et al., 2003). Our current findings suggest that rapsyn binding to the α-helical region of the loops likely mediates the constitutive rapsyn/AChR interaction, as we found that rapsyn-$CD4^{ct}$-βE10 association occurred independent of agrin signaling in muscle. However, as rapsyn can bind the α-helix on 3 subunits (ie. α, β, ε), it could also provide a site for the regulated interaction of additional rapsyn, perhaps mobilized from a free pool or activated by post-translational modification. Note that this would not be detectable in our experiments where we only expressed a single β loop fragment in muscle. Alternatively, regulated interaction might reflect rapsyn binding to a distinct site on the AChR, or recruitment of another scaffolding protein such as APC (Wang et al., 2003) that enhances rapsyn/AChR association.

Together, our findings demonstrate that rapsyn interacts with the AChR by binding an α-helical structural motif conserved between the α, β, and ε subunit loops. This provides 4 potential binding sites per receptor, although rapsyn binds preferentially to the β and ε subunit loops. Regulated binding of additional rapsyn to these sites could provide a mechanism to drive clustering and/or anchoring of the AChR in the postsynaptic membrane.

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Figure 1. Construction and expression of CD4-AChR subunit loop chimeras

(A) Schematic showing construction of the chimeric proteins consisting of CD4 extracellular and transmembrane domains fused to the major intracellular loop of the AChR subunits. **(B)** Alignment of the amino acid sequences for each of the subunits shows little sequence similarity in the intracellular loop region (* denotes identical residues; : & . denote conserved and semi-conserved substitutions, respectively). Sequences in the black boxes show the predicted α-helices in subunit loops. **(C)** CD4-loop constructs expressed in COS cells were immunoprecipitated and immunoblotted with anti-CD4 antibody. All CD4- subunit loop chimeras were expressed at similar levels. CD4-β loop was also detected by mAb124 that recognizes an epitope in the β loop, and CD4-δ by mAb88b that recognizes the δ loop.

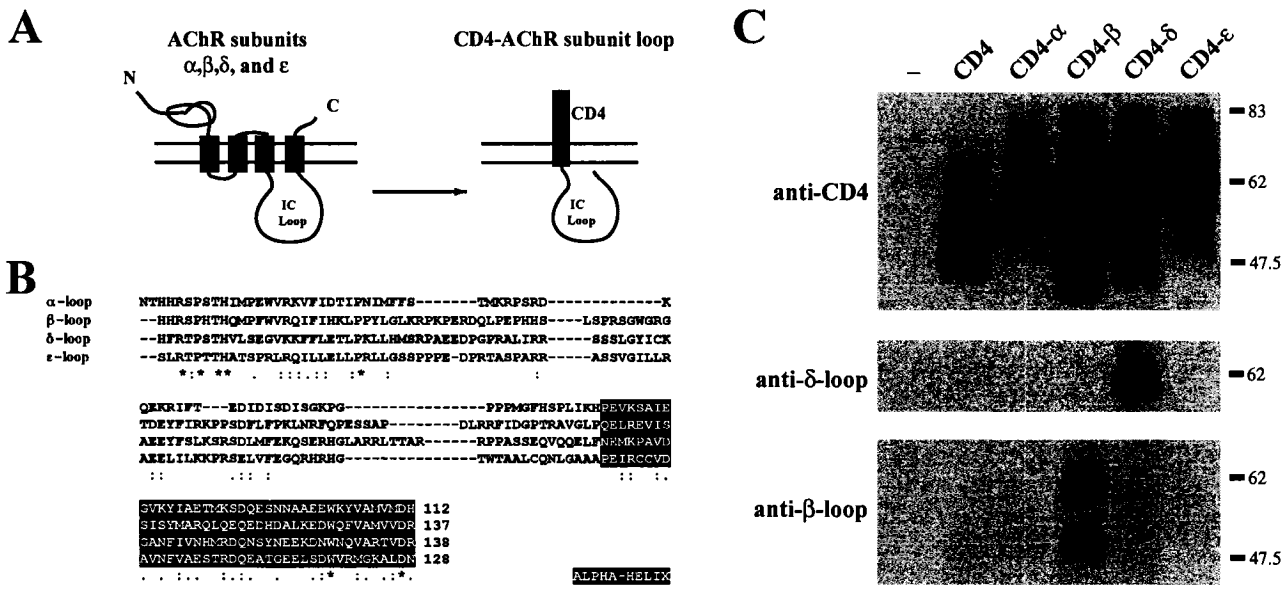

A
AChR subunits
α,β,δ, and ε
CD4-AChR subunit loop
N
C
CD4
IC Loop
IC Loop
B
α-loop
β-loop
δ-loop
ε-loop
ALPHA-HELIX
C
–
CD4
CD4-α
CD4-β
CD4-δ
CD4-ε
anti-CD4
anti-δ-loop
anti-β-loop
83
62
47.5
62
62
47.5

Figure 2. Rapsyn clusters CD4-α, -β, and -ε loops in COS cells

(A) CD4-loop chimeras were expressed in COS cells alone or together with rapsyn, and then the cells were immunostained for surface-expressed CD4-loops and for rapsyn. Expressed alone, CD4 and all of the CD4- subunit loops were diffusely distributed on the cell surface. When co-expressed with rapsyn, CD4-α, -β, and -ε loops coclustered with rapsyn aggregates, but CD4 and CD4-δ loop remained diffusely distributed. Scale bar = 50μm. **(B)** Quantification of rapsyn-induced clustering of the CD4- chimeras, showing the percentage of co-expressing cells that had strong or weak CD4-loop clusters (see methods). The highest levels of rapsyn-induced clustering occurred with CD4-β loop, followed by CD4-ε and then CD4-α (mean±SEM, n=3; * $p<0.05$ by Student's t-test).

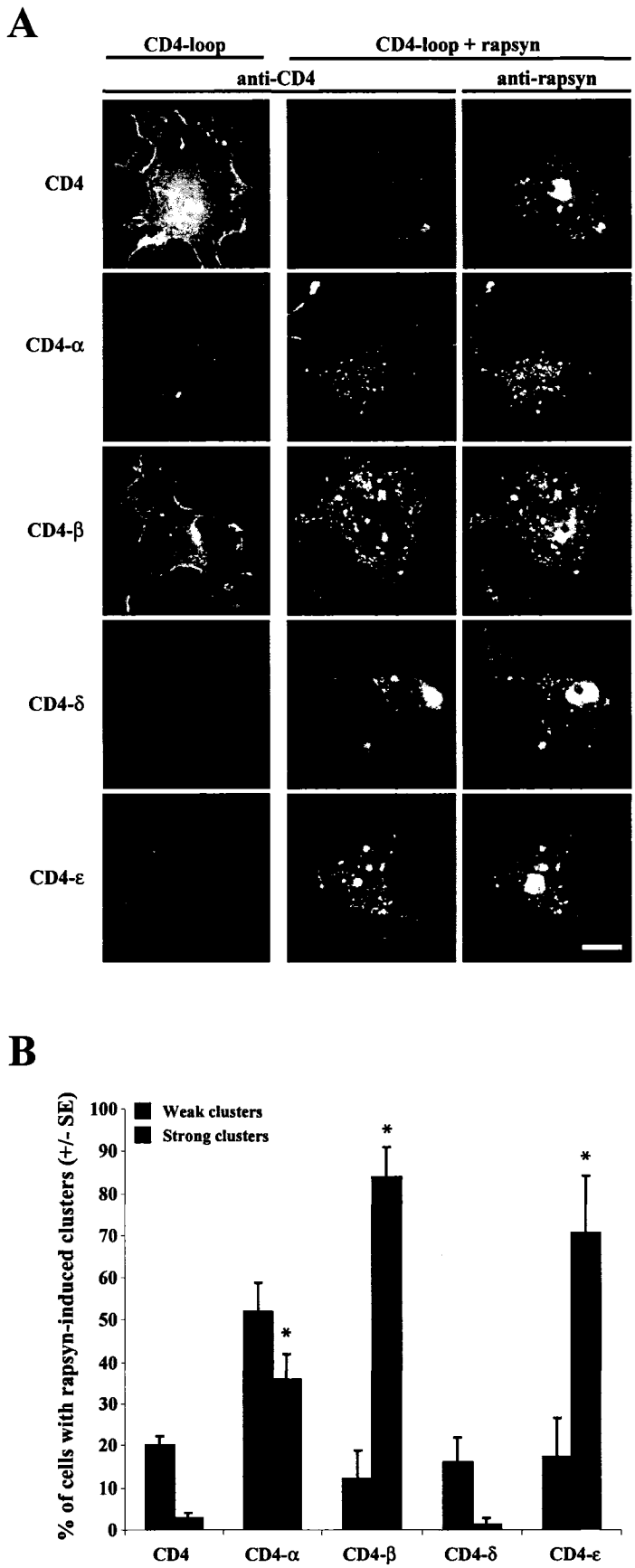
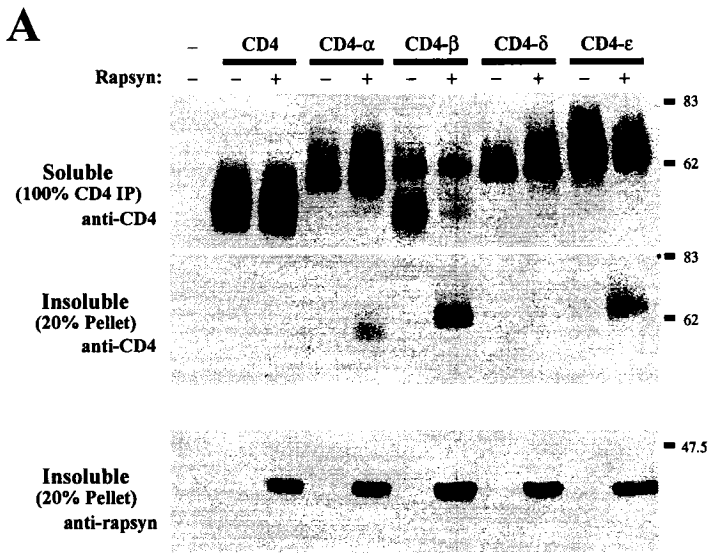
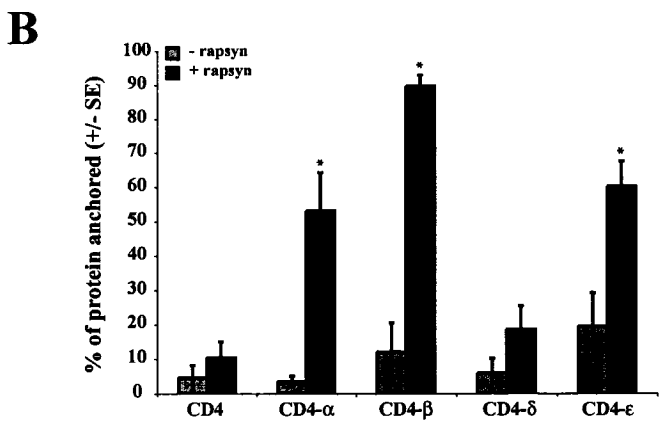

A
CD4-loop
CD4-loop + rapsyn
anti-CD4
anti-rapsyn
CD4
CD4-α
CD4-β
CD4-δ
CD4-ε
B
% of cells with rapsyn-induced clusters (+/- SE)
Weak clusters
Strong clusters
100
90
80
70
60
50
40
30
20
10
0
*
*
*
CD4
CD4-α
CD4-β
CD4-δ
CD4-ε

Figure 3. Rapsyn cytoskeletally anchors CD4-α, -β and -ε loops

(A) CD4- subunit loops were expressed in COS cells alone or together with rapsyn. The transfected cells were then extracted in 1%Triton X100-containing buffer, and the distribution of the CD4-loop chimeras in the soluble and insoluble fractions was determined by immunoblotting with CD4 antibody. Expressed alone, CD4 and all the CD4-loops were readily extracted and recovered in the soluble fraction. When co-expressed with rapsyn, however, a significant amount of CD4-β loop, and to a lesser extent CD4-α and -ε loops, were retained in the insoluble, cytoskeletal fraction. The pellet fraction was also immunoblotted for rapsyn, showing that equal levels were present in each of the transfections. **(B)** Quantification of the percentage of each CD4-loop protein retained in the insoluble fraction. Rapsyn anchored ~90% of CD4-β loop and 50-60% of CD4-α and -ε loops (n=5). In contrast, the extractability of CD4 and CD-δ were not significantly affected by rapsyn. **(C)** Quantification of total protein levels, showing that rapsyn induced three- and nine-fold increases in CD4-α and –β loop protein levels, respectively (n=5; * $p<0.05$ Student's t-test). Total levels of CD4, CD4-δ loop or CD4-ε loop were not significantly altered by rapsyn co-expression.


A
–
CD4
CD4-α
CD4-β
CD4-δ
CD4-ε
Rapsyn:
– – + – + – + – + – +
Soluble
(100% CD4 IP)
anti-CD4
83
62
Insoluble
(20% Pellet)
anti-CD4
83
62
Insoluble
(20% Pellet)
anti-rapsyn
47.5


B
- rapsyn
+ rapsyn
% of protein anchored (+/- SE)
100
90
80
70
60
50
40
30
20
10
0
*
*
*
CD4
CD4-α
CD4-β
CD4-δ
CD4-ε

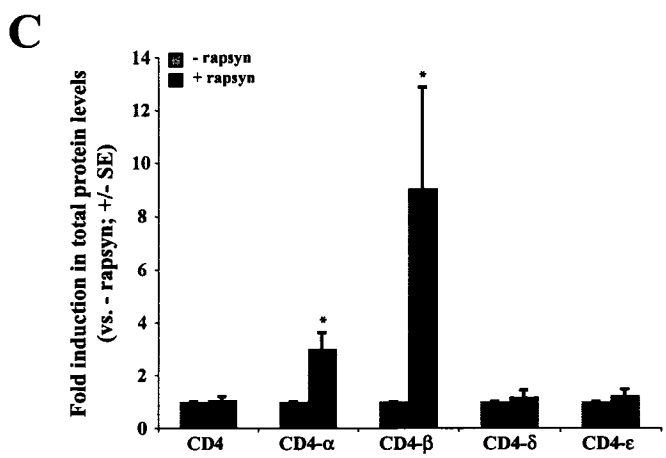

C
- rapsyn
+ rapsyn
Fold induction in total protein levels
(vs. - rapsyn; +/- SE)
14
12
10
8
6
4
2
0
*
*
CD4
CD4-α
CD4-β
CD4-δ
CD4-ε

Figure 4. Rapsyn interacts with the β loop exon10 region

(**A**) CD4-βE9 and CD4-βE10 chimeras were expressed in COS cells alone or together with rapsyn. Rapsyn induced robust clustering of CD4-βE10, but not CD4-βE9. (**B**) Rapsyn-induced anchoring of CD4-βE9 and -βE10 was assayed by extracting transfected COS cells in 1%Triton X-100-containing buffer and immunoblotting the soluble and pellet fractions with anti-CD4 antibody. With rapsyn co-expression, significant amounts of CD4-β loop and CD4-βE10 were retained in the insoluble, cytoskeletal fraction, whereas CD4 and CD-βE9 were recovered entirely in the soluble fraction. The pellet fraction was immunoblotted for rapsyn to show equivalent protein levels. (**C**) Quantification of the percentage of each protein retained in the insoluble fraction. Rapsyn anchored ~70% of CD4-β loop and ~40% of CD4-βE10 ($n=3$; $^*p<0.05$ by Student's t-test). In contrast, the extractability of CD4 and CD4-βE9 were not significantly affected by rapsyn.

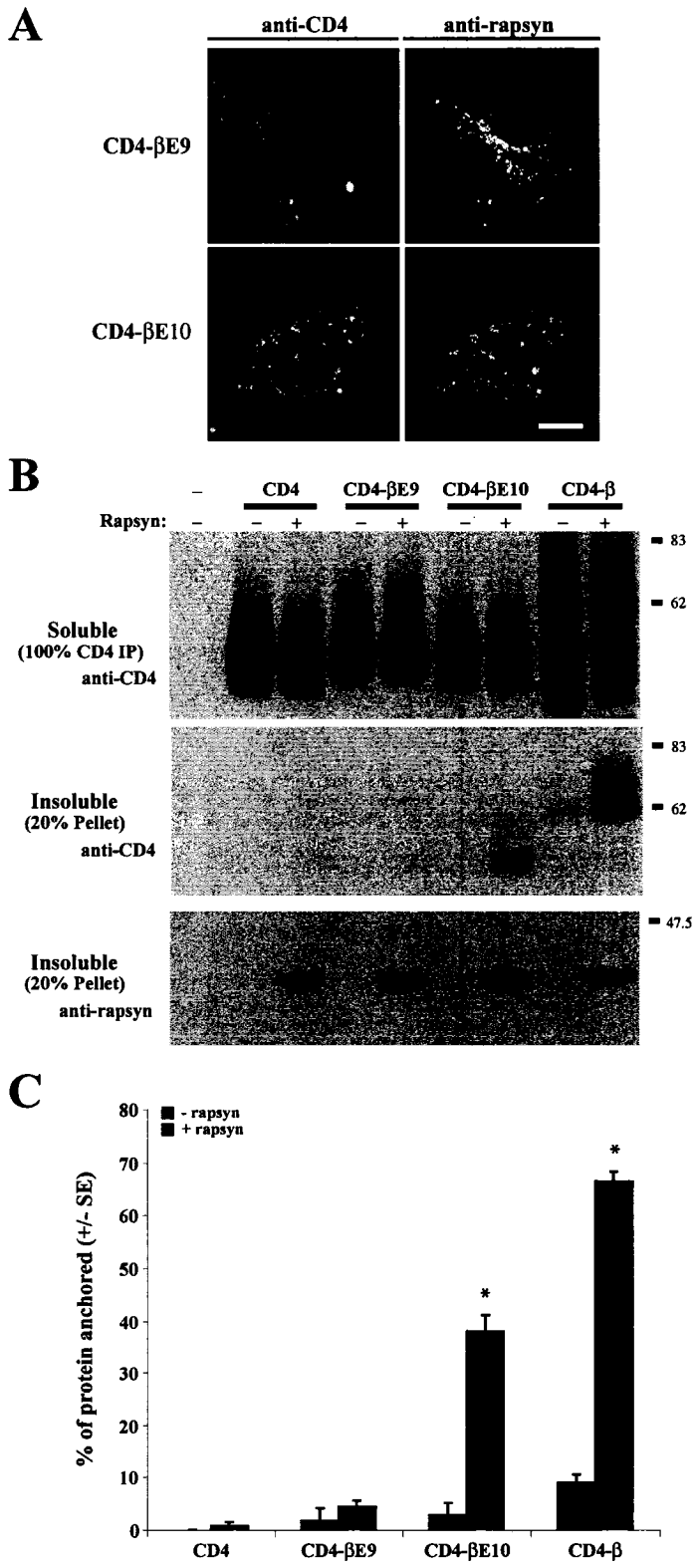
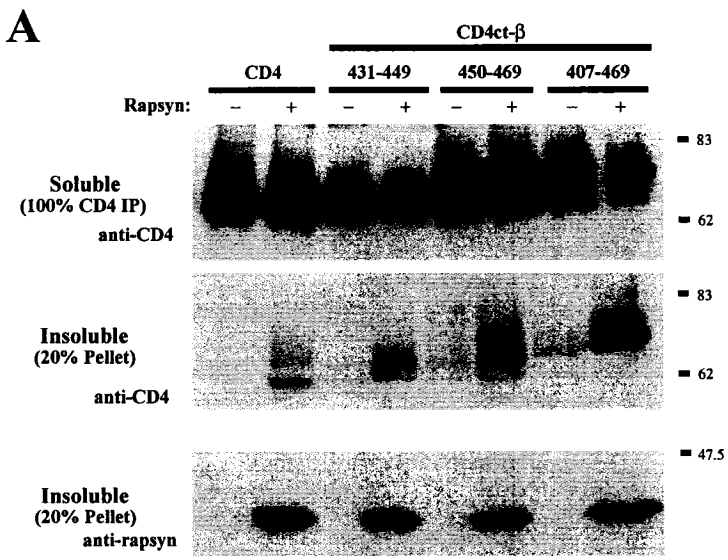

A
anti-CD4
anti-rapsyn
CD4-βE9
CD4-βE10
B
–
CD4
CD4-βE9
CD4-βE10
CD4-β
Rapsyn:
– – + – + – + – +
83
62
Soluble
(100% CD4 IP)
anti-CD4
83
62
Insoluble
(20% Pellet)
anti-CD4
47.5
Insoluble
(20% Pellet)
anti-rapsyn
C
- rapsyn
+ rapsyn
% of protein anchored (+/- SE)
80
70
60
50
40
30
20
10
0
*
*
CD4
CD4-βE9
CD4-βE10
CD4-β

Figure 5. Rapsyn anchors the α-helical region of β loop

(A) Rapsyn interaction with α-helical region was assayed by extracting in 1%Triton-containing buffer and immunoblotting the soluble and pellet fractions with anti-CD4 antibody. Rapsyn anchored both the first and second halves of the α-helix ($CD4^{ct}$-β430-449 and $CD4^{ct}$-β450-469), although to a lesser extent than the complete helix ($CD4^{ct}$-β407-469). **(B)** Quantification of the percentage of each protein retained in the pellet fraction. Rapsyn anchored ~30% and ~55% of $CD4^{ct}$-β430-449 and -β450-469, respectively, as compared to ~80% of $CD4^{ct}$-βE10 (407-469). In contrast, the extractability of CD4 was not significantly affected by rapsyn (n=6-7; * $p<0.05$ by Student's t-test).


A
CD4ct-β
CD4
431-449
450-469
407-469
Rapsyn:
– + – + – + – +
Soluble
(100% CD4 IP)
anti-CD4
83
62
Insoluble
(20% Pellet)
anti-CD4
83
62
Insoluble
(20% Pellet)
anti-rapsyn
47.5

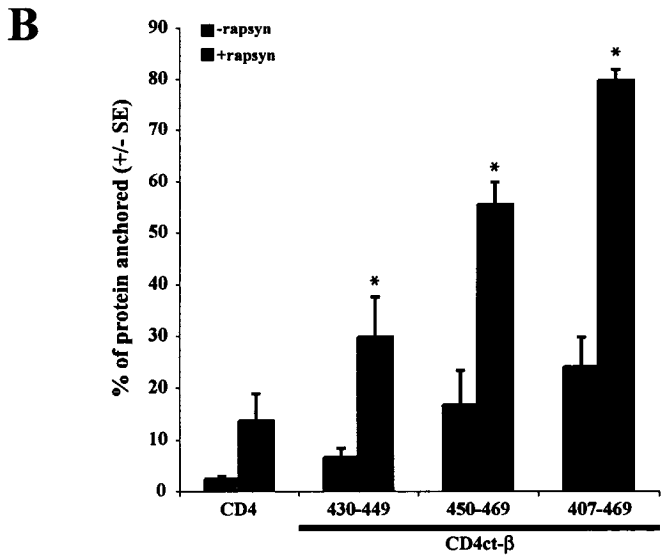
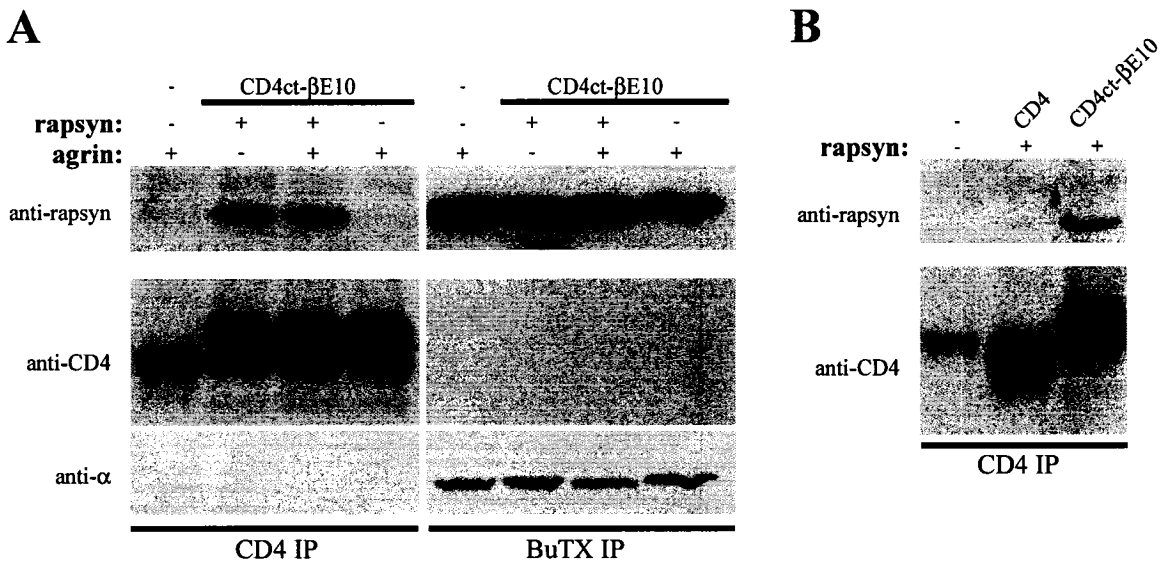

B
-rapsyn
+rapsyn
% of protein anchored (+/- SE)
90
80
70
60
50
40
30
20
10
0
*
*
*
CD4
430-449
450-469
407-469
CD4ct-β

Figure 6. Rapsyn interacts with CD4-βE10 in muscle.

(**A**) $CD4^{ct}$-βE10 was expressed in Sol8 myotubes, either alone or with additional rapsyn. $CD4^{ct}$-βE10 and AChR were then isolated from muscle cell lysates and rapsyn association assayed by immunoblotting with a rapsyn polyclonal antibody. Rapsyn was readily co-immunoprecipitated together with $CD4^{ct}$-βE10 when they were co-expressed, but only trace amounts of endogenous rapsyn associated with $CD4^{ct}$-βE10 expressed alone. Treatment with agrin also increased the interaction of rapsyn and the endogenous AChR, but had no effect on rapsyn - $CD4^{ct}$-βE10 association. Reprobing with CD4 and AChR α subunit (mAb210) antibodies confirms that $CD4^{ct}$-βE10 does not associate with the AChR. (**B**) Rapsyn co-immunoprecipitated with CD4ct-βE10 but not CD4.


A
-
CD4ct-βE10
-
CD4ct-βE10
rapsyn:
-
+
+
-
-
+
+
-
agrin:
+
-
+
+
+
-
+
+
anti-rapsyn
anti-CD4
anti-α
CD4 IP
BuTX IP
B
CD4
CD4ct-βE10
rapsyn:
-
+
+
anti-rapsyn
anti-CD4
CD4 IP

While rapsyn is indispensable for postsynaptic clustering of AChR at the NMJ (Gautam et al., 1995), it is presently unclear whether additional proteins interact with the receptor and contribute to its synaptic localization. In chapter 3, we identify an AChR motif that is sufficient for agrin-induced clustering in muscle, apparently via interaction with an additional (as yet unidentified) scaffolding protein.

Chapter III

A motif in the β subunit intracellular loop contributes to postsynaptic clustering of the acetylcholine receptor via a phosphorylation-dependent protein interaction

Lucia S. Borges, Sergey Yechikov, Young Lee and Michael Ferns

Submitted

ABSTRACT

At the neuromuscular junction, the acetylcholine receptor is specifically localized in the postsynaptic membrane via interactions with scaffolding proteins, regulated in large part by motoneuron-derived agrin. However, the protein binding sites on the acetylcholine receptor (AChR) that mediate receptor clustering are poorly defined. Here, we define domains of the AChR sufficient for agrin-induced clustering using chimeric proteins consisting of CD4 fused to the AChR subunit intracellular loops. In transfected muscle cells, we find that agrin selectively clustered CD4 chimeras containing the major intracellular loop of only the β subunit. By making further deletions, we define a β loop sequence centered on a conserved tyrosine (Y390) that is sufficient for clustering (amino acids 370-406). Overexpression of CD4 chimeras encompassing this region inhibited clustering of the endogenous AChR. Moreover, agrin-induced phosphorylation and clustering of the β loop fragment was abolished by mutation of Y390. Surprisingly, mutation of Y390 did not affect constitutive or regulated AChR-rapsyn interactions, nor did rapsyn detectably interact with phosphorylated CD4-βE9. Together, these findings suggest that novel agrin-regulated, phosphorylation-dependent interactions with the β loop motif help mediate postsynaptic clustering of the AChR.

INTRODUCTION

Rapid signaling at neuronal synapses is mediated by ligand-gated ion channels, which are concentrated in the postsynaptic membrane beneath the nerve terminal. The density of neurotransmitter receptors is a key determinant of synaptic strength and function, and thus, the specific localization of receptors is critical for synapse formation, maintenance and plasticity. Receptor localization is mediated by direct and indirect interactions with scaffolding proteins that link the receptors to the postsynaptic cytoskeleton, with these interactions being regulated by specific trans-synaptic signals. For example, at the developing neuromuscular junction, a motoneuron-derived factor called agrin stably aggregates the acetylcholine receptor at nascent synaptic contacts. Indeed, nerve-muscle contacts in agrin null mice lack stable AChR aggregates and are consequently non-functional, resulting in perinatal lethality. In addition, recombinant agrin is sufficient to induce as well as stabilize AChR clusters in cultured myotubes (reviewed in (Sanes and Lichtman, 2001; Burden, 2002; Kummer et al., 2006). Agrin localizes the AChR by signaling via the muscle-specific kinase (MuSK) and several elements of the MuSK signaling pathway have recently been identified (reviewed in (Strochlic et al., 2005)). However, it remains unclear how MuSK signaling regulates the interaction of the AChR with scaffolding proteins that localize it in the postsynaptic membrane.

One known scaffolding protein involved in AChR localization is an intracellular, membrane associated protein called rapsyn. Rapsyn colocalizes precisely with the AChR at developing neuromuscular junctions (Froehner et al., 1981; Burden, 1985; Noakes et al., 1993) and at agrin-induced clusters in cultured myotubes (Wallace, 1989). Moreover, rapsyn aggregates the AChR when they are co-expressed in heterologous cells (Froehner

et al., 1990; Phillips et al., 1991), and the AChR fails to cluster at neuromuscular synapses in rapsyn null mice (Gautam et al., 1995). Surprisingly, however, it is unclear where rapsyn binds on the AChR or how their interaction is regulated by agrin. Similarly, it is unclear whether other scaffolding and/or adaptor proteins also bind and help localize the AChR. To identify the critical interaction(s) responsible for AChR localization, we have first defined the subunits and domains of the AChR sufficient for postsynaptic receptor clustering. Using chimeric proteins consisting of CD4 fused to each of the AChR subunit intracellular loops, we identify a region in the β subunit loop that is sufficient for clustering. Agrin-induced clustering of this β subunit domain is phosphorylation-dependent and overexpression inhibits clustering of the endogenous AChR. Thus, agrin-regulated protein interactions with this β loop motif contribute to postsynaptic clustering of the AChR.

MATERIALS AND METHODS

Cell culture and transfection

Sol8 and C2 mouse muscle cells were maintained in DMEM-HI, supplemented with 20% fetal bovine serum, 100U/ml of penicillin-streptomycin, and 2mM L-glutamine. When confluent, the cells were incubated with fusion medium (DMEM-HI supplemented with 5% horse serum and 2mM L-glutamine) to induce formation of myotubes. For immunostaining experiments, the cells were grown on 8-well chamber slides (Nalge Nunc Inc. Rochester, NY), precoated with 0.1% Gelatin in the case of Sol8 cells (w/v; Fisher Scientific, Pittsburgh, PA), and the myoblasts were transfected at ~90% confluency using FuGENE6 (Roche, Indianapolis, IN). For biochemical experiments, cells were grown in 10cm dishes and transfected using the $CaPO_4$ method.

COS cells were maintained in DMEM-HI with 10% fetal bovine serum. They were grown on 10cm dishes and transfected at ~85-90% confluency using the $CaPO_4$ method.

CD4- subunit loop constructs

To generate the CD4 chimeras a Bgl II site was introduced by site directed mutagenesis at the end of the transmembrane domain of mouse CD4 in the pUC vector. The intracellular domain of CD4 was then excised with BglII and XbaI (in the 3' polylinker) and the large intracellular loops of the AChR subunits were ligated into this site. The loops were obtained by PCR of mouse cDNAs for each of the AChR subunits. Similarly, we used PCR to generate fragments of the β subunit loop. PCR based mutagenesis was performed to replace the β subunit loop tyrosine-390 residue with a phenylalanine (Y390F) using the QuickChange kit (Stratagene, La Jolla, CA). All the

CD4/loop constructs were then subcloned into the mammalian expression vector pcDNA3 and sequenced for accuracy.

Immunostaining and clustering assay

For immunostaining of transfected Sol8 and C2 muscle cells, surface AChR and CD4 chimeras were detected by incubating live myotubes with Alexa 594-conjugated α-bungarotoxin (Invitrogen-Molecular Probes, Carlsbad, CA) and anti-CD4 antibody H129.19 (BD Biosciences-Pharmingen, San Jose, CA) for 15 minutes. After washing, the myotubes were fixed with 2% paraformaldehyde for 20 min, blocked with serum, and then incubated with Alexa 488-conjugated anti-rat secondary antibody. Identical results were obtained using either Sol8 or C2 myotubes, and when the transfected cultures were fixed prior to antibody labeling. The labeled culture slides were viewed with a Zeiss Axioplan 2 IE fluorescence microscope and Plan-Apo 63x oil objective (NA 1.40), and digital images acquired using an Axiocam MRM camera and Axiovision software. In some experiments, we also used a Nikon Eclipse E600 microscope, CFI Plan Apo 60x oil objective (NA 1.4), and Optronics camera. To quantify agrin-induced clustering of CD4-subunit loops, we selected random fields and then scored all CD4-positive myotubes according to whether or not they had surface CD4 clusters colocalizing with AChR clusters. The counts were made from 3 to 6 experiments for each CD4- AChR subunit loop chimeric protein.

For the competition experiment, we used C2 myotube cultures and determined the expression levels of the CD4-chimeras (CD4, CD4-β382-406, CD4-β370-406, and CD4-ε/β370-406) by immunoprecipitating them from transfected myotubes and

immunoblotting with antibodies to the β subunit E9 region (mAb124) and to CD4. Blotting with mAb124 showed the relative expression of the chimeric constructs to that of the endogenous AChR, with the exception of CD4-β382-406 that lacks part of the mAb124 epitope. Consequently, the relative expression levels of the chimeric proteins were determined by immunoblotting for CD4. The intensity of the western blot signals was quantified using ImageGauge V4.22 software (FujiFilm, Cypress, CA), and for both mAb124 and CD4 blots, levels were normalized to CD4-β370-406. This allowed us to compare the expression levels of all chimeric proteins to the endogenous AChR. To quantify agrin-induced clustering, we selected random fields and then counted the number of AChR clusters in 125 μm segments of all CD4-positive myotubes. All transfected cultures were coded and counted blind. The number of clusters per myotube segment was normalized to that in CD4 expressing myotubes for each experiment and data collected from 8 independent experiments.

Protein extraction, immunoprecipitation and western blotting

For Sol8 transfections, cells were washed, scraped off and pelleted in ice cold PBS. They were then re-suspended in extraction buffer (0.5% Triton X-100, 25mM Tris, 25mM glycine, 150mM NaCl, 5mM EDTA and the protease inhibitors PMSF, benzamidine, N-ethylmaleimide, and $Na_2S_4O_6$) and incubated for 10 minutes on ice, after which the insoluble proteins were pelleted by centrifugation at 16,100g for 4 minutes. The CD4 chimeras and 142-tagged β subunit containing AChR were immunoprecipitated from the soluble fraction with monoclonal antibody GK1.5 (BD Biosciences-Pharmingen, San Jose, CA) and mAb142 (Sigma), respectively; both antibodies were chemically cross-

linked to protein G-agarose (Invitrogen, Carlsbad, CA). Endogenous AChR was then isolated using biotinylated α-bungarotoxin and streptavidin-agarose (Invitrogen-Molecular Probes, Carlsbad, CA). After resuspending in 2X protein loading buffer (SDS, glycerol, 10% β-mercaptoethanol and bromophenol blue), the samples were boiled and separated on 10% polyacrylamide gels. We then immunoblotted with monoclonal antibodies H129.19 against CD4 and mAb124 to AChR β subunit, followed by HRP-conjugated anti-rat secondary antibody (Amersham, Piscataway, NJ) and visualization with enhanced chemiluminescence. Phosphorylation of CD4-β loop chimeras were confirmed by immunoblotting with JH-1360, an antibody specific for phosphorylated β subunit Y390 residue (Gillespie et al., 1996), followed by incubation with HRP-conjugated anti-rabbit antibody (Amersham, Piscataway, NJ). To detect co-immunoprecipitated rapsyn, we immunoblotted with rabbit polyclonal antibody B5668. The blots were then reprobed with antibody JH-1360 to determine levels of AChR β subunit phosphorylation, and with mAb210 against the AChR α subunit. The intensity of the western blot signals was quantified using ImageGauge V4.22 software (Fuji Film, Cypress, CA).

For COS cell transfections, the cells were extracted as above and CD4-β loop chimeras were immunoprecipitated from the soluble fraction with anti-CD4 antibody (GK1.5) chemically conjugated to protein G-agarose. In addition, the insoluble pellet was resuspended directly in 200μl of 2X protein loading buffer. Samples were immunoblotted with monoclonal antibody H129.19 to detect CD4. The blots were reprobed with polyclonal antibody B5668 to detect rapsyn, and monoclonal antibody 9E10 to detect myc-MuSK

RESULTS

Direct and indirect interactions with scaffolding proteins are thought to localize the AChR in the postsynaptic muscle membrane (Banks et al., 2003); however, the relevant binding sites on the AChR are poorly defined. To identify the domains in the AChR that mediate its synaptic localization, we generated chimeric constructs where the CD4 extracellular and transmembrane domains were fused with the major intracellular loops of each of the AChR subunits (Fig 1A). This allowed surface expression and enabled us to test each subunit loop individually and determine whether it contains motifs involved in agrin-induced clustering of the receptor. To first confirm expression, the CD4- subunit chimeras were expressed in Sol8 myotubes by transient transfection and then immunoprecipitated and immunoblotted using an antibody to the CD4 extracellular domain (Fig 1B). We found that all the CD4-loop chimeras were robustly expressed and ran at the expected molecular weights. Moreover, reprobing with antibodies to the AChR α or β subunits demonstrated that the CD4-loop chimeras did not assemble together with endogenously expressed receptor subunits (data not shown and see figures 4 & 5).

To assay agrin-induced clustering of the CD4-subunit chimeras, transfected cultures were treated with neural (Z+) agrin (~200pM for 16 hr) and immunostained for surface CD4 and AChR. Strikingly, we observed clustering of only CD4-β subunit loop (Fig 2), which aggregated together with the endogenous AChR in ~70% of expressing myotubes (Table 1). In contrast, CD4 and CD4- $\alpha,\gamma,\delta,\varepsilon$ loops all remained diffusely distributed on the myotube surface despite normal clustering of the endogenous AChR. CD4-δ loop was not as well expressed on the cell surface as the other chimeras, however, and we cannot exclude the possibility that it clusters at low levels. In addition, we tested a CD4-ε loop

chimera that included the 4th transmembrane domain and c-terminus (CD4-εCT) and found that it also failed to cluster in response to agrin treatment (Fig 2, Table 1). Thus, only the β subunit loop is sufficient for clustering, suggesting protein interaction motifs specific for this subunit contribute to AChR localization.

Next, we defined the region of the β subunit loop that mediates clustering by making further deletions or substitutions. First, we tested CD4-chimeras containing the first (amino acids 333-406) or second halves (aa 407-469) of the β loop; these are encoded primarily by exons 9 and 10 of the subunit gene, respectively (Fig 3C). We found that agrin induced robust clustering of CD4-βE9 (in ~75% of expressing myotubes), compared to essentially no clustering of CD4-βE10 (Fig 3A, Table 1). Identical results were obtained when we substituted the βE9 and βE10 regions into the corresponding region of ε loop; only CD4-βE9/ε coclustered with the endogenous AChR (Fig 3A). We then tested CD4 chimeras with further deletions within the E9 region and found efficient clustering of CD4-β370-406 but no detectable clustering of CD4-β382-406 (Fig 3B). Similarly, we observed clustering of CD4-ε/β370-406, where β370-406 was substituted into the corresponding region of ε loop. These findings identify a 37 amino-acid sequence in the β subunit loop that is sufficient for agrin-induced clustering.

If protein interactions with the β loop E9 region are important for AChR clustering, then overexpression of CD4-β loop chimeras should inhibit clustering of the endogenous AChR by competing for the requisite binding protein. To test this, we transfected myotubes with CD4-β370-406 and -ε/β370-406, which cluster, and CD4-β382-406 and CD4, which do not cluster. We selected these chimeras as they were expressed at higher levels than CD4-β loop or βE9 and encompass just the region sufficient for aggregation.

To first assay their expression levels, we immunoprecipitated the CD4 chimeras from myotube extracts and immunoblotted with antibodies to CD4 and the β subunit loop E9 region (mAb124); this showed that all were expressed at levels approximately 2.5 - 3 times that of the endogenous AChR (Fig 4A,B). When we then counted agrin-induced AChR clusters (in blinded fashion), we found that cluster number was decreased ~40% in myotubes expressing CD4-β370-406 and -ε/β370-406, as compared to control myotubes expressing CD4 or CD4-β382-406 (Fig 4C,D; $p<0.01$, Student's t-test, n=8). As we quantified AChR clustering in all transfected myotubes, this understates the more pronounced decrease observed in myotubes with high levels of CD4-β370-406 and -ε/β370-406 expression. Moreover, we found that AChR aggregates in these myotubes were often fragmented (Fig 4C), suggesting that the coalescence of microaggregates into dense, stable AChR clusters is impaired in the presence of the competing β370-406 fragment. Agrin-induced AChR clustering is inhibited therefore only by β loop E9 fragments that aggregate, indicating that this region contributes significantly to clustering of the AChR.

Interestingly, the β loop E9 region contains a conserved tyrosine residue (Y390) that is phosphorylated after agrin treatment and that has been implicated in receptor localization (Wallace et al., 1991; Borges and Ferns, 2001). To test for phosphorylation of this site in the CD4-β loop chimeras, they were immunoprecipitated from extracts of control and agrin-treated myotubes, and then immunoblotted with Y390 phospho-specific antibody (Gillespie et al., 1996). We observed agrin-induced phosphorylation of CD4- β loop, βE9 and βE9/ε within 1 hour of agrin treatment (Fig 5A), prior to discernable receptor aggregation. We next tested whether phosphorylation was required for clustering

by mutating tyrosine 390 to phenylalanine (Y390F); this eliminated phosphorylation without affecting the expression levels of the chimera (Fig 5B). Intriguingly, mutation of Y390 abolished agrin-induced clustering of all 3 chimeras (CD4-β loop, βE9, βE9/ε), which remained diffusely distributed despite extensive clustering of the endogenous AChR (Fig 5C, Table 1). These findings demonstrate that agrin-induced phosphorylation of Y390 is essential for clustering of the isolated β loop region. Although the δ subunit loop contains a similar tyrosine (Wagner et al., 1991) that is also phosphorylated by agrin (Mittaud et al., 2001; Mohamed et al., 2001), we did not observe any clustering of CD4-δ loop (Table 1). Similarly, we did not detect clustering of a CD4-ε loop construct where we introduced a tyrosine residue at this position (data not shown). This suggests that additional, flanking sequences in the β loop are required for phosphorylation-dependent aggregation.

These findings, together with our previous results (Chapter 2), indicate that there are two distinct types of protein interactions that contribute to AChR clustering: a regulated interaction via the β subunit exon9 region that requires agrin-induced phosphorylation of Y390, and a constitutive rapsyn interaction with the α-helical region of the subunit intracellular loops (Chapter 2; Lee and Ferns, in preparation). One intriguing possibility is that rapsyn mediates both forms of interaction, binding to two distinct sites on the receptor. Consistent with this, agrin treatment of cultured myotubes increases both AChR β subunit phosphorylation (Wallace et al., 1991; Ferns et al., 1996) and the amount of rapsyn co-immunoprecipitated with the AChR (Moransard et al., 2003). This indicates that rapsyn/AChR association is regulated, with agrin inducing either new or higher affinity rapsyn/AChR complexes by some form of post-translational

modification. We tested, therefore, whether rapsyn/AChR association is regulated by β subunit Y390 phosphorylation and if this involves rapsyn binding to the β loop exon9 region.

To investigate whether agrin regulates AChR-rapsyn association through β subunit phosphorylation, we first tested whether the two events are temporally correlated. We treated cultured C2 myotubes with Z+ agrin for one hour, isolated surface AChR, and then assayed β subunit phosphorylation and rapsyn association by western blot analysis. Even at the early timepoint of 1 hr, we find that agrin increased the amount of rapsyn co-immunoprecipitated with surface AChR, along with β subunit phosphorylation (Fig 6A). In additional time-course experiments, the two events were detectable after 5 minutes of agrin stimulation and reached maximum levels after 40 minutes (personal communication, C. Fuhrer). Thus, the agrin-induced increase in AChR-rapsyn association correlates closely with β subunit phosphorylation.

Next, we tested whether AChR β subunit phosphorylation is required for rapsyn association in muscle cells by mutating the relevant tyrosine (Y390). To do this, we expressed wild type or Y390F forms of 142-tagged β subunit in Sol8 myotubes by transient transfection; these tagged β subunits assemble together with the endogenous subunits to form AChR that is expressed on the myotube surface (Borges and Ferns, 2001). After agrin treatment for 1 hr, AChR containing tagged and endogenous β subunit were sequentially isolated from cell extracts and then immunoblotted for associated rapsyn (Fig 6B). As previously observed (Moransard et al., 2003), agrin increased the amount of rapsyn co-immunoprecipitated with endogenous AChR by ~1.5 fold. Similarly, we found that agrin increased rapsyn association with AChR containing both tagged wild

type and Y390F β subunit (by ~1.4 and ~1.6 fold, respectively; Fig 6C). The amount of rapsyn associated with the AChR prior to agrin treatment was also not significantly different between βwt- and βY390F-AChR. Thus, phosphorylation of β subunit Y390 is not required for either the constitutive or agrin-regulated interaction of rapsyn with the AChR.

In addition, we tested for interaction of rapsyn with phosphorylated β subunit exon9 in heterologous cells. To do this, we expressed rapsyn and CD4-βE9 in COS cells together with MuSK to induce βY390 phosphorylation (Chapter 4) and then assayed rapsyn-induced anchoring. As shown in Chapter 2, rapsyn significantly reduced the detergent extractability of CD4-β loop that contains the α-helical structure; however, we did not detect any rapsyn-induced anchoring of CD4-βE9 in the insoluble, cytoskeletal fraction (Fig 7). Together, these findings suggest that the phosphorylated β subunit "clustering motif" does not interact with rapsyn, but rather with an as yet-unidentified binding partner that contributes to AChR localization in the postsynaptic muscle membrane.

DISCUSSION

Together, our findings identify a novel β subunit-specific motif that is sufficient for targeting to postsynaptic AChR clusters. The β loop 37 amino acid sequence was the sole region of the AChR that mediated postsynaptic localization and its addition to CD4 or substitution into ε loop resulted in efficient agrin-induced clustering of these chimeras. Moreover, we found that overexpression of two different CD4-βE9 chimeras both inhibited agrin-induced clustering of the endogenous AChR. Thus, we propose that protein interactions with this region are necessary for postsynaptic localization of the AChR and likely occur with relatively high stoichiometry.

The protein interacting with the β loop motif is unclear but appears distinct from previously identified AChR binding and clustering proteins such as rapsyn and APC. Indeed, rapsyn can cluster all of the receptor subunits in heterologous cells (Maimone and Merlie, 1993; Huebsch and Maimone, 2003) and consistent with this, we have found that rapsyn interacts with the α-helix, a secondary structure in the second half of the loop that is conserved between the different subunits (Lee and Ferns, in preparation). We also failed to detect any association between rapsyn and CD4-βE9 in heterologous cells, even under conditions where Y390 was phosphorylated. Similarly, APC binds the non-overlapping, E10 region of β subunit loop and this domain is not sufficient for clustering (Wang et al., 2003). Thus, an additional, as yet unidentified, protein likely interacts with the β E9 region (Fig 8A). Potentially, this protein could act in parallel with rapsyn to localize the AChR, perhaps with an independent and complementary function. For example, rapsyn might laterally aggregate the AChR in the membrane whereas the second protein might vertically anchor the receptor to the postsynaptic cytoskeleton.

Alternatively, it could act together with rapsyn, as part of a tertiary complex (see model in Fig 8B).

Interestingly, the β loop "clustering" domain is centered on a tyrosine residue (Y390) and surrounding sequence of ~20 amino acids that is highly conserved between species. We find that agrin induces phosphorylation of this site in the CD4-β loop chimeras, and strikingly, its mutation abolished all clustering; thus, protein interactions with the β loop E9 region are phosphorylation-dependent. Consistent with this, previous studies have shown that synaptic AChR contains phosphorylated β subunit and that agrin induces a pronounced phosphorylation of the AChR β subunit in cultured myotubes (Wallace et al., 1991; Ferns et al., 1996). This agrin-induced phosphorylation occurs rapidly on surface AChR, prior to discernable aggregation (Borges and Ferns, 2001), and correlates with linkage of the receptor to the cytoskeleton (Wallace, 1992; Wallace, 1995). Moreover, mutation of β subunit Y390 inhibits agrin-induced anchoring and clustering of the AChR (Borges and Ferns, 2001), although clustering is not abolished as in the CD4-β loop chimeras. This difference is probably due to rapsyn (or another scaffolding protein) being able to bind other domains in the AChR but not the isolated β loop.

Surprisingly, our results indicate that agrin-induced β subunit phosphorylation does not regulate AChR-rapsyn interaction, despite the close temporal correlation between the two events. Indeed, we found no interaction between rapsyn and phosphorylated CD4-βE9 in hetelogous cells, and mutation of Y390 did not affect rapsyn-AChR interaction in muscle cells. Presumably, therefore, the agrin-induced increase in rapsyn-AChR association is regulated by posttranslational modification of

another AChR subunit, rapsyn, or an additional interacting protein. One candidate is tyrosine phosphorylation of the AChR δ subunit, which is also tightly correlated with agrin-induced receptor clustering (Mittaud et al., 2001). In fact, agrin-induced phosphorylation of the AChR β and δ subunits occur with similar time-courses and are inhibited by the same pharmacological agents (Mittaud et al., 2001; Mohamed et al., 2001). Another candidate is rapsyn, which we find is preferentially tyrosine-phosphorylated downstream of MuSK, at least in heterologous cells (Lee and Ferns, in preparation; Chapter 4). Finally, regulation of other interacting proteins such as APC could also increase the stoichiometry or affinity of rapsyn /AChR interaction.

We propose, therefore, that AChR localization is not mediated solely by rapsyn, and that at least two different protein interactions combine to cluster the AChR in the postsynaptic membrane: rapsyn binding to the α helix of multiple subunit loops (Huebsch and Maimone, 2003)(Lee and Ferns, in preparation) and regulated binding of an unidentified protein to the phosphorylated β subunit loop E9 region (Fig 8B). While it is unclear whether rapsyn's interaction with the AChR is primarily constitutive or regulated (Fuhrer et al., 1999; Marangi et al., 2001; Moransard et al., 2003; Zhu et al., 2006), our findings clearly indicate that protein binding to the β E9 region is phosphorylation-dependent and regulated by agrin signaling. Consequently, agrin-induced phosphorylation of β Y390 - and the resulting protein binding - probably serves as an important regulatory step in the pathway that localizes the AChR at the synapse. During initial synaptogenesis, agrin-induced binding of a scaffolding protein to the β subunit loop likely helps induce and stabilize AChR clusters in the postsynaptic membrane. Similarly, at mature synapses,

it could provide a mechanism by which agrin signaling regulates AChR levels in the postsynaptic membrane, thus modulating synaptic strength.

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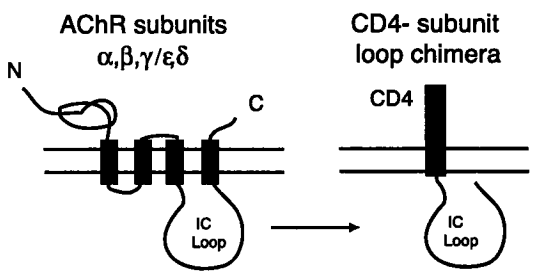
Figure 1. Construction and expression of CD4-AChR subunit loop chimeras. (A) Schematic showing AChR subunit topology and construction of CD4-subunit intracellular (IC) loop chimeras. (B) CD4-subunit loops were expressed transiently in Sol8 myotubes and then immunoprecipitated from cell extracts and immunoblotted with an antibody to the CD4 extracellular domain. All CD4-subunit loop chimeras were robustly expressed.

A


AChR subunits
α,β,γ/ε,δ
N
C
IC
Loop
CD4- subunit
loop chimera
CD4
IC
Loop

B

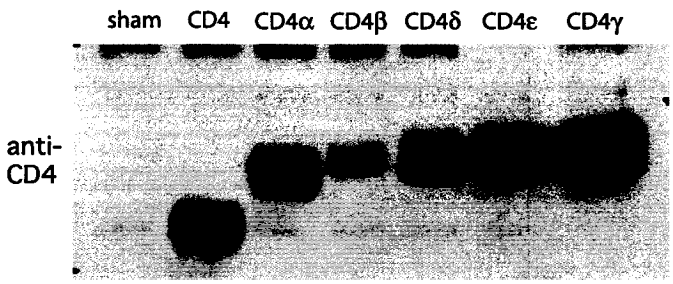

sham
CD4
CD4α
CD4β
CD4δ
CD4ε
CD4γ
anti-
CD4

Figure 2. Agrin induces clustering of only CD4-β loop. Sol8 myotubes transfected with CD4-subunit loop chimeras were treated with agrin for 16 hrs, and then immunostained for surface CD4 and AChR. Only CD4-β loop coclustered with the AChR in response to agrin treatment (arrows). CD4 and all other CD4-subunit loops remained diffusely distributed on the myotube surface (CD4-γ and δ loops are not shown but see Table 1). CD4-εCT denotes an additional chimera that includes the IC loop, 4th transmembrane domain and C-terminus, which also failed to cluster. Scale bar = 20 μm.

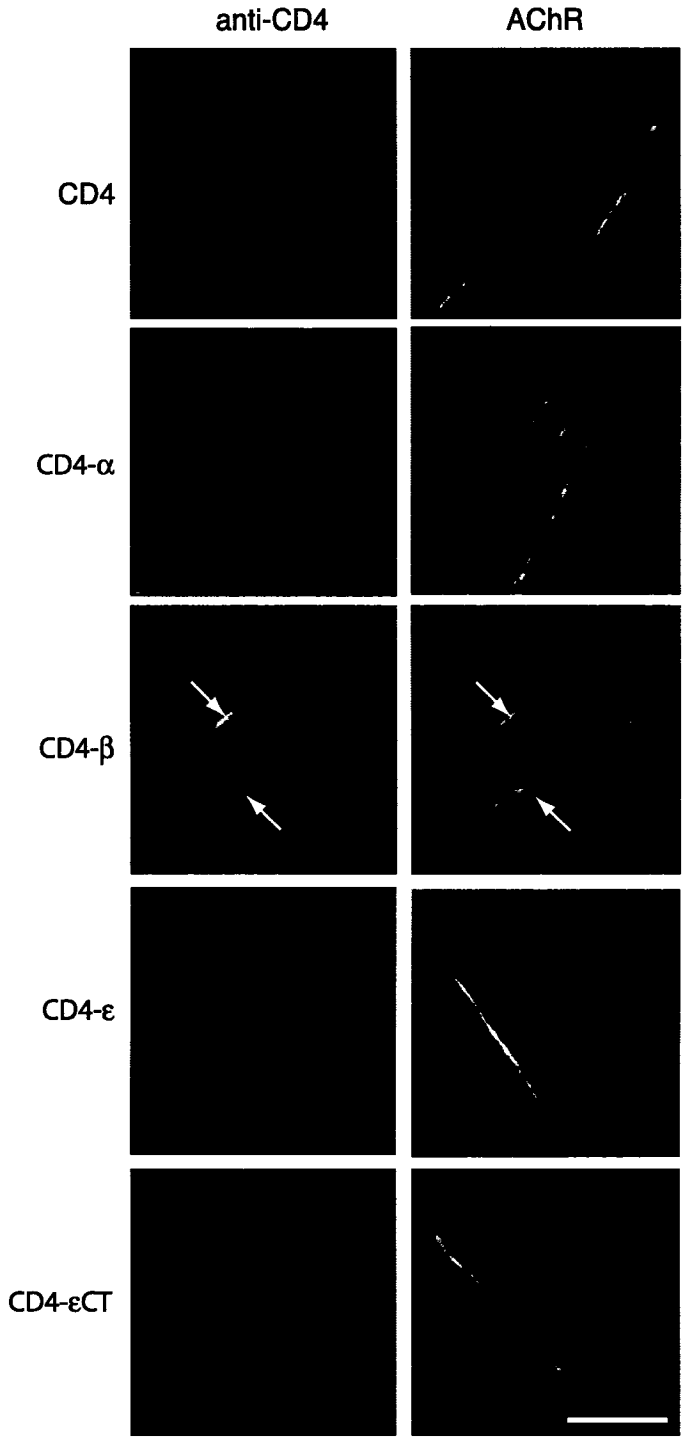

anti-CD4
AChR
CD4
CD4-α
CD4-β
CD4-ε
CD4-εCT

Figure 3. A 37 amino acid motif in the β subunit loop is sufficient for clustering.

(A) CD4- loop constructs were generated with just the βE9 or E10 regions, or with βE9 or E10 substituted into the corresponding region of ε loop. Agrin induced clustering of CD4-βE9 and -βE9/ε (arrows) but not -βE10 or ε/βE10 chimeras. (B) CD4 chimeras with further deletions in E9 region; agrin induced clustering of CD4-β370-406 but not β382-406. (C) Schematic shows β loop structure (with E9 and E10 regions) and indicates the sequence (370-406) found to be sufficient for clustering. Y390 denotes the conserved tyrosine phosphorylation site and the thickened line represents the predicted α-helix.

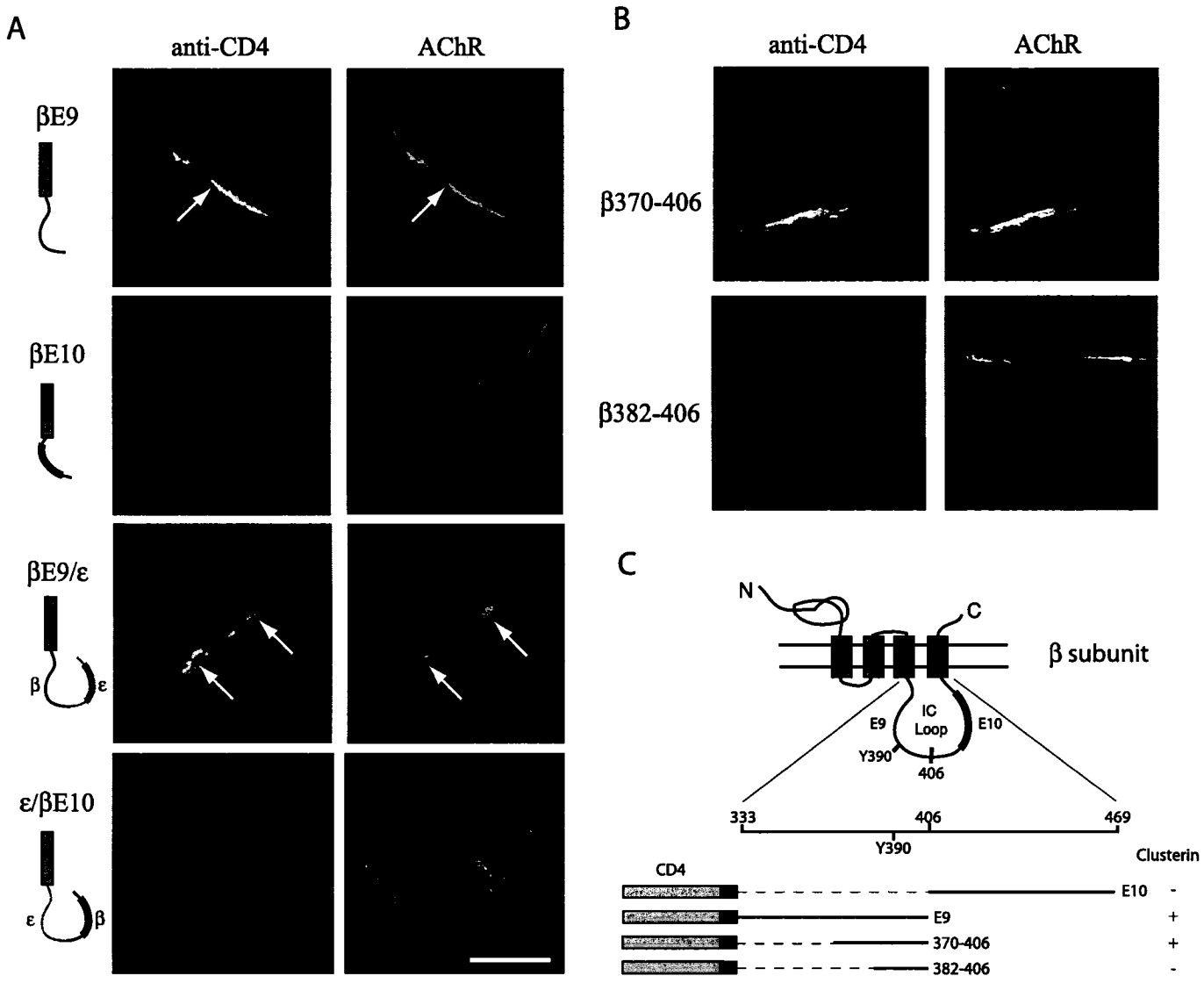

A
anti-CD4
AChR
βE9
βE10
βE9/ε
β
ε
ε/βE10
ε
β
B
anti-CD4
AChR
β370-406
β382-406
C
N
C
β subunit
E9
IC
Loop
E10
Y390
406
333
406
469
Y390
Clusterin
CD4
E10 -
E9 +
370-406 +
382-406 -

Figure 4. Overexpression of CD4-β loop chimeras inhibits agrin-induced clustering of the endogenous AChR. (A) CD4-β loop chimeras and AChR were isolated from extracts of transfected Sol8 myotubes and their levels compared by immunoblotting for CD4 and the β loop (with mAb124). Note that the mAb124 epitope is partially lost in β382-406. (B) Quantification of the CD4 and mAb124 immunoblots shows that the 3 CD4-β loop constructs were expressed at similar levels, approximately 2.5-3 times that of the endogenous AChR. (C) Immunostaining for surface CD4 and AChR shows that receptor clustering is inhibited in myotubes overexpressing CD4-β370-406. The AChR clusters are also fragmented and less intensely stained (arrows) as compared to those in untransfected or CD4 expressing myotubes (arrowheads). (D) Quantification of the number of agrin-induced AChR clusters in myotubes overexpressing CD4 or CD4-β loop chimeras. Compared to CD4-expressing control myotubes, the number of AChR clusters was significantly reduced (~40%) in myotubes expressing β370-406 and ε/β370-406 but not β382-406 which does not aggregate ($p<0.01$, Student's t-test, n=8).

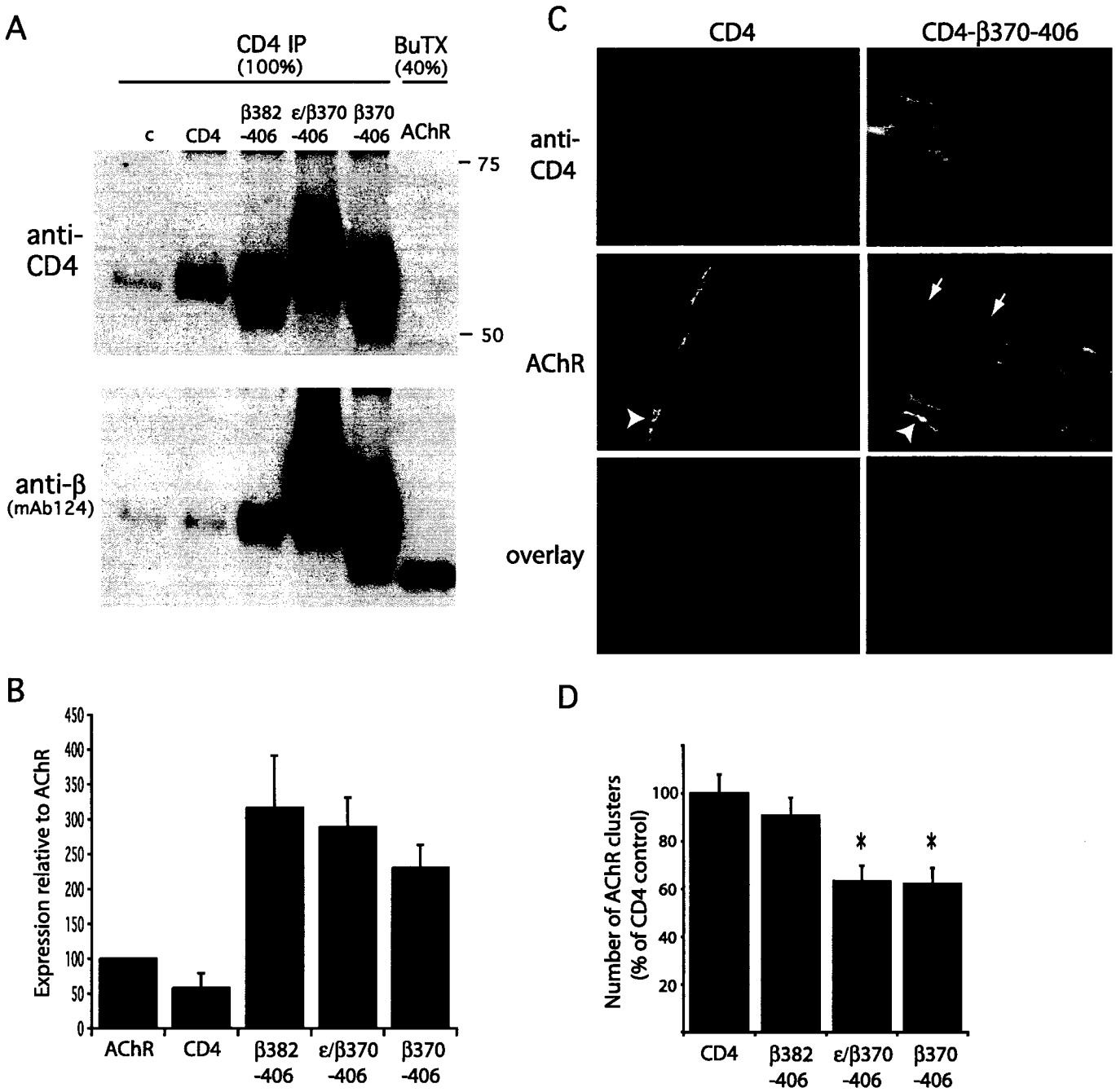

A
CD4 IP
(100%)
BuTX
(40%)
c
CD4
β382
-406
ε/β370
-406
β370
-406
AChR
anti-
CD4
anti-β
(mAb124)
75
50
C
CD4
CD4-β370-406
anti-
CD4
AChR
overlay
B
Expression relative to AChR
450
400
350
300
250
200
150
100
50
0
AChR
CD4
β382
-406
ε/β370
-406
β370
-406
D
Number of AChR clusters
(% of CD4 control)
100
80
60
40
20
*
*
CD4
β382
-406
ε/β370
-406
β370
-406

Figure 5. Agrin-induced clustering of CD4-β loop chimeras is phosphorylation-dependent. (A) CD4-β loop constructs were immunoprecipitated from extracts of control and agrin-treated Sol8 myotubes (C-$Ag_{4,8}$,200 pM, ~1hr) and immunoblotted with a phospho-specific antibody to Y390. Agrin induced phosphorylation of CD4-β, βE9 and βE9/ε. Reprobing with mAb124 to the β loop confirmed similar levels of chimeric proteins in each of the immunoprecipitations. (B) Sol8 myotubes were transfected with CD4-βE9 and CD4-βE9 Y390F where the tyrosine was mutated to phenylalanine. Mutation of Y390 eliminated phosphorylation without affecting expression levels as shown by reprobing with an antibody to CD4. (C) Agrin-induced clustering of CD4-β, βE9, and βE9/ε was abolished by Y390F mutation. Scale bar = 20 μm.

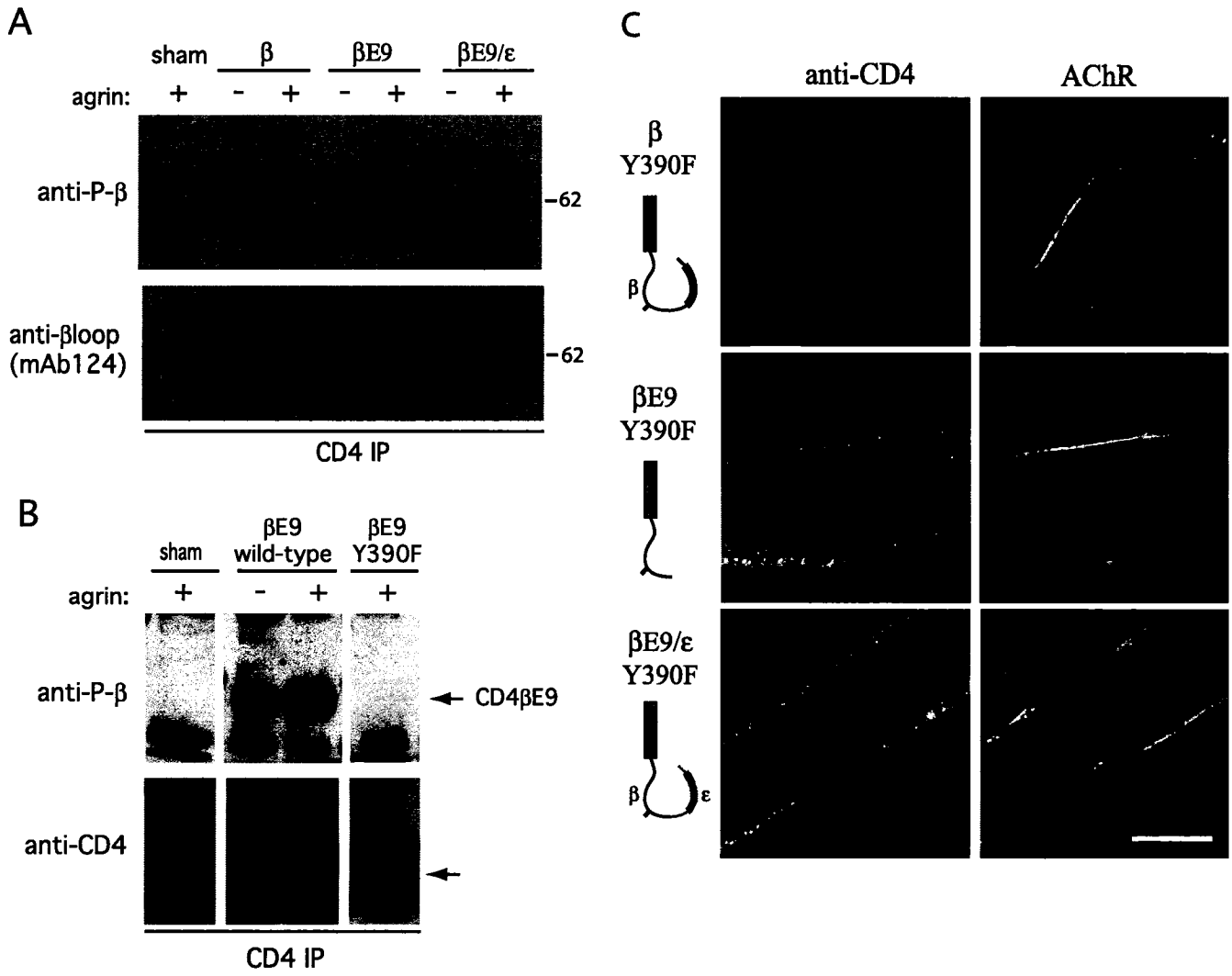
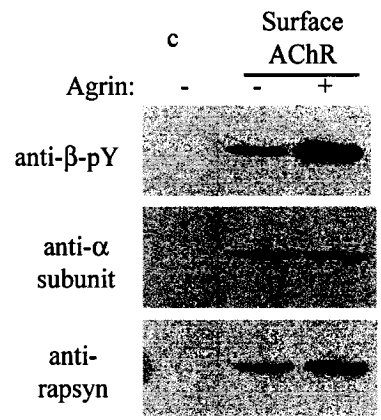
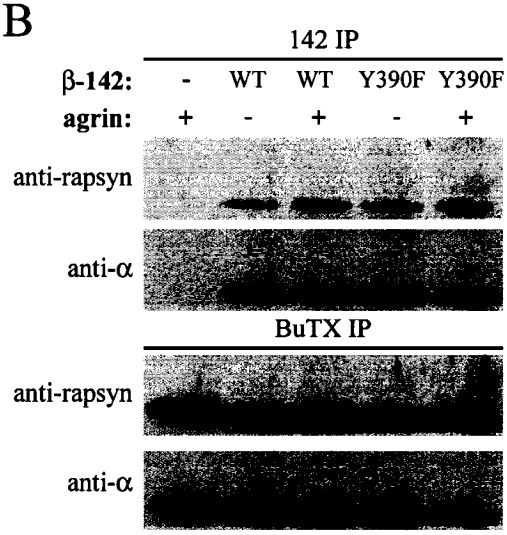

A
sham
β
βE9
βE9/ε
agrin:
+ - + - + - +
anti-P-β
-62
anti-βloop
(mAb124)
-62
CD4 IP
B
sham
βE9
wild-type
βE9
Y390F
agrin:
+ - + +
anti-P-β
CD4βE9
anti-CD4
CD4 IP
C
anti-CD4
AChR
β
Y390F
β
βE9
Y390F
βE9/ε
Y390F
β
ε

Figure 6. AChR-rapsyn association does not require phosphorylation of β subunit Y390. (**A**) Surface and intracellular pools of AChR were isolated from control and agrin-treated Sol8 myotubes and then immunoblotted for β subunit phosphorylation and for associated rapsyn. Agrin treatment for 1 hr (C-$Ag_{4,8}$, 200 pM) increased both the amount of rapsyn that co-immunoprecipitated with the AChR and β subunit phosphorylation in surface receptor. (**B**) Wildtype or Y390F forms of 142-tagged β subunit were expressed in Sol8 myotubes by transient transfection. AChR containing tagged and endogenous β subunit were sequentially isolated from control and agrin-treated myotubes (C-$Ag_{4,8}$, 200 pM, 1 hr) and then immunoblotted for rapsyn. Similar amounts or rapsyn were detected in association with βwt and βY390F-AChR in both control and agrin-treated myotubes. (**C**) Quantification of agrin-induced increase in AChR-associated rapsyn. Agrin treatment caused a similar, ~1.5 fold increase in rapsyn association with AChR containing βwt-142 βY390F-142 and endogenous β subunit.

A


c
Surface AChR
Agrin: - - +
anti-β-pY
anti-α subunit
anti-rapsyn

B


142 IP
β-142: - WT WT Y390F Y390F
agrin: + - + - +
anti-rapsyn
anti-α
BuTX IP
anti-rapsyn
anti-α

C

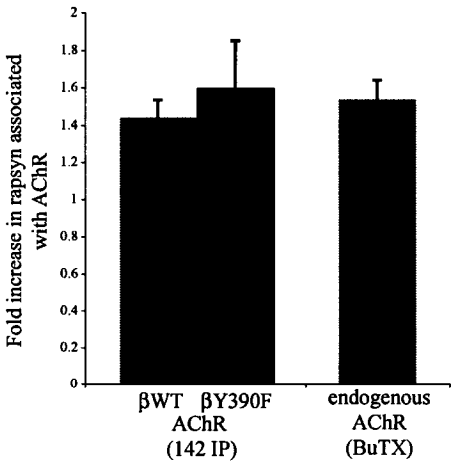

Fold increase in rapsyn associated with AChR
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
βWT βY390F AChR (142 IP)
endogenous AChR (BuTX)

Figure 7. Rapsyn does not anchor phosphorylated CD4-βE9. CD4-βE9 was co-expressed in COS cells together with rapsyn and MuSK (to induce phosphorylation of Y390). After extraction in 0.5% Triton X-100 containing buffer, the soluble and pellet fractions were immunoblotted with anti-CD4 antibody. Rapsyn significantly reduced the detergent extractability of CD4 β loop containing the α-helix, with a significant proportion being found in the cytoskeletal pellet fraction. In contrast, rapsyn did not affect the detergent extractability of CD4-βE9, which was detected solely in the soluble fraction.

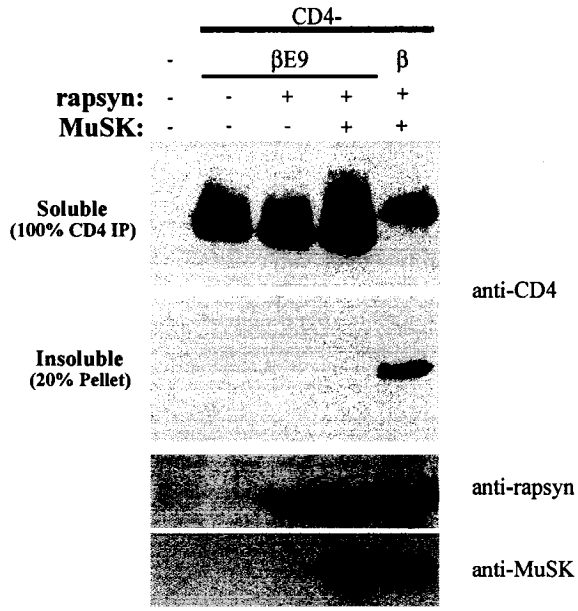

CD4-
-
βE9
β
rapsyn:
MuSK:
Soluble
(100% CD4 IP)
Insoluble
(20% Pellet)
anti-CD4
anti-rapsyn
anti-MuSK

Figure 8. Model for protein interactions localizing the AChR in the postsynaptic membrane. (A) A motif in the β subunit E9 region (centered on Y390) is sufficient for clustering, binding an as yet unidentified protein in a phosphorylation-dependent manner. Note that rapsyn and APC bind elsewhere, to the α-helix in the C-terminal portion of the intracellular loop. (B) Our findings suggest that AChR localization is mediated by multiple interactions, including rapsyn binding to the α-helix of multiple subunit loops and regulated binding of an additional protein to the β loop E9 region. Potentially, the two scaffolding proteins could act in parallel with distinct functions such as clustering and anchoring, or together as part of a tertiary complex. As agrin-induced phosphorylation of Y390 is required for binding of the scaffolding protein to β E9, this likely helps regulates the postsynaptic localization of the AChR. For simplicity, membrane tethering of rapsyn and APC binding have been omitted.

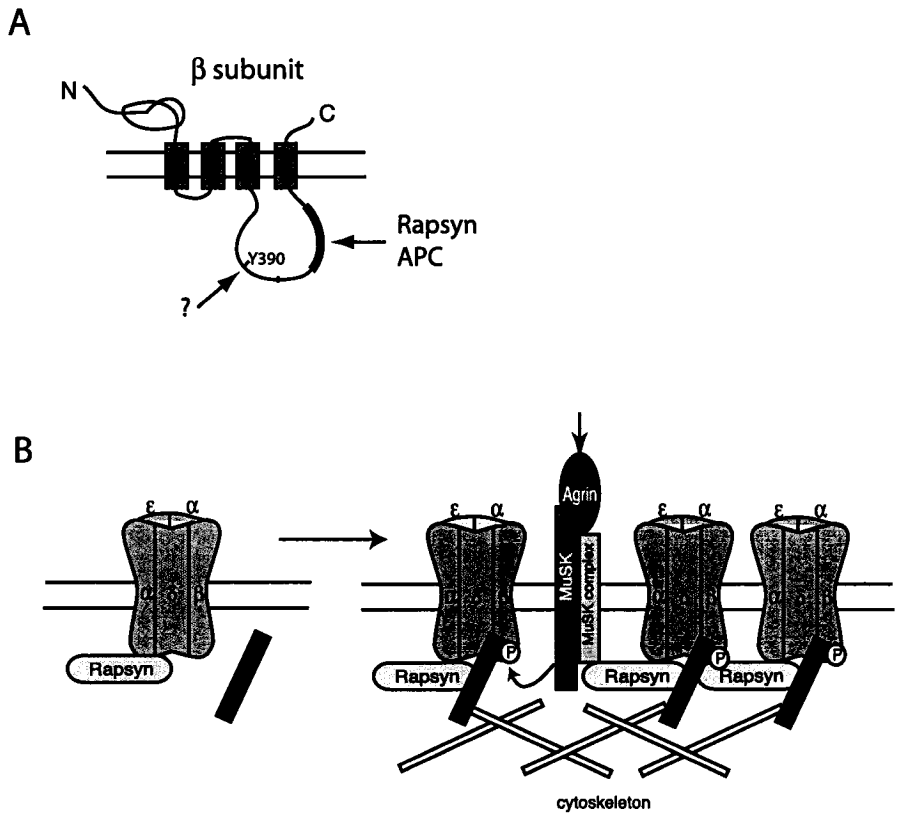

A
β subunit
N
C
Rapsyn
APC
Y390
?
B
ε
α
Agrin
MuSK
MuSK complex
Rapsyn
P
cytoskeleton

Table 1. Quantification of agrin-induced clustering of the different CD4-subunit loops and β loop constructs. CD4-chimera positive myotubes were selected in random fields and the number of CD4 clusters that colocalized with AChR clusters was then counted. Agrin induced robust clustering of CD4-β loop, βE9, βE9/ε and β370-406 with approximately 75% of the expressing myotubes having clusters. Mutation of Y390 abolished clustering of these constructs, and all other CD4-loops showed only background levels of colocalization with AChR clusters.

Table 1

CD4-chimera	# of experiments	# of myotubes counted	total # of clusters	% of myotubes with clusters
CD4	5	80	1	1.25
CD4-α	5	88	0	0
CD4-β	6	94	111	73.4
CD4-γ	3	57	0	0
CD4-δ	5	63	2	3
CD4-ε	5	75	0	0
CD4-βE9	6	117	168	74.3
CD4-β/ε	6	111	171	77.5
CD4-βE10	5	84	2	2.3
CD4-βloopY390F	6	100	2	1
CD4-βE9Y390F	3	57	1	1.75
CD4-β/εY390F	3	53	0	0

Table 1. Quantification of agrin-induced clustering of the different CD4-subunit loops and β loop constructs. Random CD4-chimera positive myotubes were selected and the number of CD4 clusters that colocalized with AChR clusters was then counted. Agrin induced robust clustering of CD4-β loop, βE9, and β/ε, with approximately 75% of the expressing myotubes having clusters (red, last column). Mutation of Y390 abolished clustering of these constructs, and all other CD4-loops showed only background levels of colocalization with AChR clusters.

The agrin-induced AChR phosphorylation, a signaling event that regulates AChR clustering (Wallace et al., 1991; Ferns et al., 1996), is severely impaired in rapsyn null muscle (Apel et al., 1997; Mittaud et al., 2001). We hypothesized that rapsyn plays an active role early in the agrin-signaling pathway to mediate AChR phosphorylation, in addition to its functions as a critical scaffolding protein, at the developing NMJ. In chapter 4, we identify the rapsyn domain(s) that facilitates MuSK-induced AChR phosphorylation.

Chapter IV

Rapsyn C-terminal domains mediate MuSK-induced phosphorylation of the AChR

Young Lee and Michael Ferns

In preparation

ABSTRACT

At the developing vertebrate neuromuscular junction, the postsynaptic localization of the AChR is directed by agrin signaling via the MuSK receptor tyrosine kinase and requires an intracellular scaffolding protein called rapsyn. In addition to its structural role, rapsyn is also necessary for agrin-induced tyrosine phosphorylation of the AChR, which regulates some aspects of receptor localization. Here, we have investigated the molecular mechanism by which rapsyn mediates AChR phosphorylation. Using a heterologous cell system, we find that rapsyn facilitates MuSK-induced phosphorylation of both the AChR β and δ subunits. Intriguingly, MuSK also induces tyrosine phosphorylation of rapsyn itself. While phosphorylation of the β and δ subunits occurs in parallel, mutation of the relevant tyrosine in each subunit showed that their phosphorylation is not inter-independent. To then map the domains in rapsyn that activate and/or localize the tyrosine kinases phosphorylating the subunits, we tested a series of rapsyn N- and C-terminal deletion mutants for their ability to induce cellular tyrosine phosphorylation. We find that the C-terminal of rapsyn is both necessary and sufficient for tyrosine kinase activation. Moreover, deletion of just the rapsyn RING domain abolishes MuSK-induced tyrosine phosphorylation of the AChR β subunit. Together, these findings suggest that rapsyn facilitates AChR phosphorylation by binding and activating tyrosine kinases via its C-terminal domains.

INTRODUCTION

At the developing neuromuscular junction in vertebrates, several nerve-derived signals combine to specifically localize the acetylcholine receptor at postsynaptic sites (Sanes and Lichtman, 2001; Burden, 2002; Kummer et al., 2006). One essential factor is agrin, which signals via the MuSK receptor tyrosine kinase and induces and/or stabilizes clustering of the nAChR in the postsynaptic membrane (reviewed in (Kummer et al., 2006). Indeed, in agrin and MuSK knockout mice, there is no stable aggregation of AChR at nerve-muscle contacts and the mice die at birth due to an inability to move and breath (DeChiara et al., 1996; Gautam et al., 1996). Downstream of MuSK activation, an important mediator of AChR clustering is the intracellular, peripheral membrane protein, rapsyn, which associates with the AChR in the postsynaptic membrane in approximately 1:1 stoichiometry (Burden, 1985; LaRochelle and Froehner, 1986; Wallace, 1989; Noakes et al., 1993). When expressed in heterologous cells, rapsyn self-aggregates and is sufficient to cluster, anchor and stabilize the AChR. Moreover, in rapsyn null mice, there is a complete absence of AChR clusters at developing synaptic sites (Gautam et al., 1995). Together, these findings suggest that rapsyn directly binds the receptor, clustering and anchoring it in the postsynaptic membrane.

Although rapsyn mediates AChR localization, it is unclear how this is regulated by agrin signaling in muscle cells. Potentially, protein interactions underlying localization could be regulated via posttranslational modifications of the AChR, rapsyn, or additional binding proteins. Consistent with the first possibility, agrin/MuSK signaling induces rapid tyrosine phosphorylation of the AChR β and δ subunits (Mittaud et al., 2001; Mohamed et al., 2001), mediated by an intervening cytoplasmic tyrosine kinase (Fuhrer et al., 1997), perhaps of the src and/or abl families (Mohamed and Swope, 1999; Finn et al., 2003).

Phosphorylation correlates closely with reduced detergent extractability and mobility of the AChR in the membrane (Meier et al., 1995; Relan et al., 1999; Borges and Ferns, 2001), suggesting that it regulates linkage to the cytoskeleton. In addition, it precedes AChR clustering (Ferns et al., 1996) and tyrosine kinase inhibitors that block phosphorylation also block clustering (Wallace et al., 1991; Ferns et al., 1996). Most importantly, mutation of the tyrosine phosphorylation site in the β subunit abolishes agrin-induced cytoskeletal anchoring of mutant AChR in muscle cells and also impairs its aggregation (Borges and Ferns, 2001). Phosphorylation of the β subunit contributes to AChR localization, therefore, but it is unclear whether it does so by regulating rapsyn interaction (Fuhrer et al., 1999; Marangi et al., 2001; Moransard et al., 2003).

In addition to its structural role, rapsyn also functions in agrin signaling. Notably, agrin-induced phosphorylation of the AChR β and δ subunits is significantly decreased in rapsyn null myotubes (Apel et al., 1997; Mittaud et al., 2001), and rapsyn activates src family kinases in heterologous cells (Qu et al., 1996; Mohamed and Swope, 1999), resulting in tyrosine phosphorylation of multiple cellular proteins. Thus, rapsyn may facilitate MuSK-induced phosphorylation of the AChR by activating and/or localizing the relevant cytoplasmic tyrosine kinases.

In this study, we have investigated how rapsyn mediates the functionally important tyrosine phosphorylation of the AChR. We find that rapsyn mediates both β and δ subunit phosphorylation, and that its C-terminus is both necessary and sufficient for tyrosine kinase activation. In addition, we find that the rapsyn C-terminus including the RING domain is absolutely required for MuSK-induced tyrosine phosphorylation of the β

subunit. Together, our findings suggest that rapsyn mediates phosphorylation of AChR β and δ subunits by activating tyrosine kinases via its C-terminal domains.

MATERIALS AND METHODS

Expression Constructs

The pRcRSV vector was used to drive the expression of a wild-type rat MuSK construct that was myc-tagged at the N-terminus (provided by S. Burden, NYU). The mouse muscle nAChR subunits (α, β, ε, δ) were expressed using a CMV promoter in the pcDNA3 vector (Invitrogen; Carlsbad, CA). To epitope tag the β- and δ- subunit, a KpnI and a ClaI sites were introduced at C-terminal extracellular tails, and double stranded oligonucleotides that coded for the hemagglutinin (HA) and 142 epitopes were ligated into these sites, respectively. Tyrosines 390 of β subunit and 393 of δ subunit were replaced by phenylalanines using polymerase chane reaction-based site-directed mutagenesis (Quickchange Kit, Stratagene; La Jolla, CA). Rapsyn deletion mutants (H6-tagged at the C-termini) were generated through PCR, then ligated into the pMT23 vector (Reichardt Lab, UCSF).

Cell Culture and Transfection

COS cells were grown in DMEM-HI containing 10% fetal bovine serum and penicillin-streptomycin. For expression in COS cells, the cultures were transfected overnight with plasmid DNA using the calcium phosphate method (Profection kit, Promega; Madison, WI) at 85-90% confluency. The transfected COS cells were rescued in fresh growth medium the next day and collected for analysis two days post-transfection.

Assaying Phosphorylation of AChR

COS cells grown on 10cm dishes were transfected overnight with 20μg of plasmid DNA. AChR expressed in COS cells were surface labeled with biotin-conjugated α-bungarotoxin (Molecular Probes; Eugene, OR) for 1 hour, rinsed, collected in Ca^{++}/Mg^{++}-free PBS containing 1 mM sodium vanadate, and pelleted. Cell pellets were then lysed with buffer containing 1% Triton X-100 and 25 mM Tris-Glycine pH 7.5, 150 mM NaCl, 5mM EDTA, 1mM sodium vanadate, 50mM sodium fluoride and the protease inhibitors: 1 mM bisbenzimidine, 1 mM sodium tetrathionate, and 1mM PMSF. After incubation on ice for 10 minutes, the insoluble cytoskeletal fraction was pelleted and separated from detergent soluble fraction by centrifugation at 16,100 g for 4 min at 4°C. The insoluble pellet was homogenized and resuspended directly in 200μl of SDS-PAGE loading buffer. The lysates were incubated with streptavidin-agarose beads (Molecular Probes; Eugene, OR) for 1 hour to isolate pre-labeled AChR. Sequentially, MuSK expressed in the COS cells are immunoprecipitated, with anti-MuSK polyclonal antibody (gift of J. Sugiyama and Z. Hall, UCSF and NIH). The beads were pelleted and washed three to five times in a 50 mM Tris buffer containing 0.5 M NaCl. The proteins isolated on the beads were then eluted directly in 30 μl of SDS-PAGE sample buffer.

Samples were electrophoretically separated on 10% SDS-PAGE gels, and transferred onto PVDF membranes. To immunoblot for phosphorylated AChR β and δ subunits, the proteins were probed with polyclonal antibodies JH-1360 and JH-1358, respectively (Mohamed and Swope, 1999) in buffer containing 4% Blotto, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% NP40, and 0.1% Tween-20. The blots were then incubated with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Amersham Corp.; Arlington Heights, IL) and bound antibody visualized by

chemiluminescence (ECL, Amersham Corp.). The immunoblots were reprobed for the HA-tagged β and 142-tagged δ subunits with monoclonal antibodies anti-HA (Roche; Indianapolis, IN) or mAb142 (Sigma-Aldrich; St. Louis, MO), respectively, or for the α subunit using mAb210 (Babco; Berkley, CA). Reprobes for rapsyn were performed using monoclonal antibody mAb1234 (gift of S. Froehner, U. of Washington) or polyclonal antibody B5668 (generated against a peptide encompassing amino acids 133-153 of rapsyn). Reprobes for tagged MuSK were performed using anti-myc monoclonal antibody 9E10.

To quantify levels of tyrosine phosphorylation we carried out densitometric analysis of the relevant bands using Sci-Scan 5000 Bioanalysis software (USB; Cleveland, OH). In order to average several independent experiments all values were expressed as a percentage of that for cells co-expressing AChR, MuSK and rapsyn.

Assaying total cellular phosphorylation

COS cells grown on 6cm dishes were transfected overnight with 9μg of plasmid DNA. After 1 day of expression, cells were rinsed, scraped off and pelleted in Ca^{++}/Mg^{++}-free PBS containing 1mM sodium vanadate. The cells were then lysed in 100μl of extraction buffer (1% Triton X-100, 25 mM Tris-Glycine pH 7.5, 150 mM NaCl, 5mM EDTA, 1mM sodium vanadate, 50mM sodium fluoride, 1 mM bisbenzimidine, 1 mM sodium tetrathionate, and 1mM PMSF), and the residual pellets were resuspended in 100μl of loading buffer (SDS, glycerol, 5% β-mercaptoethanol, and bromophenol blue) and boiled. 10μl of the pellet fraction were separated on 10% acrylamide gels and then immunoblotted with monoclonal anti-phosphotyrosine antibodies 4G10 and PY20 to

determine the level of cellular protein tyrosine phosphorylation. To determine rapsyn expression, a duplicate gel was blotted with mAb1234. The intensity of the western blot signal was quantified using Sci-Scan 5000 Bioanalysis (USB; Cleveland, OH). The levels of cellular tyrosine phosphorylation were represented as a percentage of rapsyn wt expressing COS cells.

RESULTS

Rapsyn facilitates MuSK-induced phosphorylation of AChR β and δ subunits

Agrin-induced phosphorylation of the AChR plays an important role in receptor clustering in the postsynaptic membrane. To investigate how rapsyn mediates this phosphorylation, we expressed the AChR in COS cells by transient transfection, either alone or in combination with rapsyn and/or MuSK. After extracting the cells with 1% Triton-containing buffer, we isolated the AChR using alpha-bungarotoxin conjugated to agarose beads and immunoblotted with antibodies specific for the phosphorylated β and δ subunits (Y390 in β and Y393 in δ) (Wagner et al., 1991; Mohamed and Swope, 1999). The non-extractable pellet fraction was also immunoblotted as rapsyn can anchor the receptor to the cytoskeleton (Phillips et al., 1993; Mohamed and Swope, 1999). The blots were then reprobed for HA-tagged β subunit, MuSK and rapsyn to confirm their levels and distribution in the soluble versus pellet fractions. In agreement with previous a report (Gillespie et al., 1996), we find that expression of MuSK and rapsyn together with the AChR resulted in significant phosphorylation of AChR β and δ subunits (Fig 1). Most of the phosphorylated receptor was also found in the non-extractable, cytoskeletal pellet fraction. Importantly, rapsyn mediated the MuSK-induced phosphorylation of the AChR, as comparatively little phosphorylation was detected when the AChR was expressed alone or with just MuSK. Thus, our results in heterologous cells mirror that in muscle where rapsyn is required for efficient agrin-induced phosphorylation of the AChR (Apel et al., 1997; Mittaud et al., 2001).

Several studies have shown β and δ subunit phosphorylation to be closely linked, both temporally and in their sensitivity to kinase blockers (Mohamed and Swope, 1999;

Mittaud et al., 2001). This could reflect parallel phosphorylation of the 2 sites by the same - or a similar - kinases. Alternatively, phosphorylation might be sequential, with phosphorylation at one site recruiting a kinase that then phosphorylates the second subunit. To distinguish these possibilities, we expressed AChR with mutations of either β subunit Y390 or δ subunit Y393 to abolish phosphorylation and tested the effect on MuSK/rapsyn-induced phosphorylation of the other subunit (Fig 2A,B). We find that mutation of β Y390 did not significantly decrease δ subunit phosphorylation compared to AChR with wild type β subunit (78 ± 6%, mean ± SEM; n=5). Likewise, mutation of δ Y393 did not affect phosphorylation of the β subunit (89 ± 3%, mean ± SEM; n=5). Thus, phosphorylation of these subunits is not interdependent but rather occur in parallel downstream of MuSK activation.

Rapsyn C-terminus is necessary and sufficient for activation of cellular tyrosine kinases

Rapsyn likely mediates MuSK-induced phosphorylation of the AChR by activating and/or localizing cellular tyrosine kinases that phosphorylate the β and δ subunits. Indeed, rapsyn activates src family kinases in heterologous cells, resulting in increased tyrosine phosphorylation of multiple cellular proteins (Qu et al., 1996; Mohamed and Swope, 1999). Moreover, agrin specifically activates src-family kinases associated with the AChR in muscle cells and this occurs in a rapsyn-dependent manner (Mittaud et al., 2001). We examined, therefore, whether rapsyn's activation of kinases is enhanced by MuSK and which domains in rapsyn are responsible. First, we expressed rapsyn and/or MuSK in COS cells and assayed phosphorylation by immunoblotting cell

lysates with anti-phosphotyrosine antibodies 4G10 and PY20. As previously reported (Qu et al., 1996; Mohamed and Swope, 1999), rapsyn alone significantly increased cellular tyrosine phosphorylation compared to controls transfected with empty vector (by ~250%; Fig 3A,B). In contrast, MuSK alone increased phosphorylation only marginally and MuSK coexpression with rapsyn did not increase overall cellular phosphorylation beyond the levels found with rapsyn alone. A notable exception, however, was that MuSK significantly increased tyrosine phosphorylation of rapsyn itself (by ~350%; Fig 3A,C). This specific phosphorylation of rapsyn suggests that its function might also be regulated by agrin-induced phosphorylation during synaptogenesis.

To then map the domains in rapsyn responsible for kinase activation, we tested a series of deletion mutants for their ability to induce cellular tyrosine phosphorylation. Full-length wild type and His6-tagged rapsyn increased levels of tyrosine phosphorylation by approximately 2.5 to 3 fold (Fig 4B). In contrast, 3 different rapsyn C-terminal deletion mutants failed to increase tyrosine phosphorylation above background levels, despite being expressed at levels equivalent to wild type rapsyn. Indeed, deletion of just the RING domain and following 10 amino acids (rapsyn 1-365) completely abolished kinase activation (Fig 4C).

We then tested whether the rapsyn C-terminus alone activated kinases and increased tyrosine phosphorylation. Strikingly, we found that 2 different rapsyn C-terminal fragments (rapsyn 158-412 and 212-412) both increased cellular tyrosine phosphorylation to levels equivalent to that seen with wild type rapsyn (Fig 4D,E). Unfortunately, further mapping of the responsible domains was not possible due to poor expression of smaller C-terminal fragments (data not shown). However, these results

demonstrate that the C-terminal domains of rapsyn are necessary and sufficient for activation of cellular tyrosine kinases.

Rapsyn C-terminus is required but not sufficient for AChR phosphorylation

Next, we investigated whether rapsyn's C-terminal domains mediate MuSK-induced phosphorylation of the AChR. To do this, we co-expressed AChR and MuSK together with rapsyn C- and N-terminal deletion mutants and then assayed tyrosine phosphorylation of the β subunit. Consistent with our previous results, we found that all 3 rapsyn C-terminal deletions abolished MuSK-induced β subunit phosphorylation (Fig 5A,B). Thus, the RING domain is required for rapsyn's ability to mediate AChR tyrosine phosphorylation. The C-terminus of rapsyn was not sufficient for AChR phosphorylation, however, as β subunit phosphorylation was undetectable with either of the rapsyn C-terminal fragments (aa 158-412 and 212-412; Fig 5C,D). N-terminal rapsyn domains are also required, therefore, likely mediating membrane targeting or self-association of rapsyn.

Finally, we tested whether MuSK-induced phosphorylation of rapsyn might (Fig 3) regulate its ability to mediate AChR phosphorylation. While numerous conserved tyrosine and serine phosphorylation consensus sites are present in rapsyn we focused on those in the C-terminal domain implicated in kinase activation. Mutation of tyrosines 336 and 363 and serines 338, 369, 383, 405 and 406 did not inhibit rapsyn-mediated phosphorylation of the AChR β subunit however (Fig 6).

DISCUSSION

In this study, we investigated how rapsyn mediates agrin-induced phosphorylation of the AChR, a signaling event that regulates postsynaptic localization (Borges and Ferns, 2001). We show that rapsyn mediates parallel – but independent – phosphorylation of the β and δ subunits. Moreover, we find that this requires the C-terminal domains of rapsyn, which are both necessary and sufficient for tyrosine kinase activation. Thus, in addition to its structural role in localizing the AChR, rapsyn facilitates receptor phosphorylation by activating tyrosine kinases via its C-terminal domains.

Phosphorylation of AChR β and δ subunits

In muscle cells, agrin- or nerve-induced clustering of the receptor involves tyrosine phosphorylation of both the AChR β and δ subunits (Wallace et al., 1991; Ferns et al., 1996; Borges and Ferns, 2001). Following MuSK activation, phosphorylation of each subunit occurs with a similar time-course and is inhibited by the same pharmacological agents (Mittaud et al., 2001; Mohamed et al., 2001). Moreover, both β and δ subunit phosphorylation are dependent on rapsyn, with phosphorylation levels being significantly decreased in rapsyn null myotubes (Mittaud et al., 2001). However, one difference is that basal levels of phosphorylation are higher for δ than β, whereas agrin-induced phosphorylation occurs with higher stoichiometry on β (Meier et al., 1995; Mittaud et al., 2001). Agrin-induced phosphorylation of the β and δ subunits could also serve different functions. Mutation of the β subunit tyrosine phosphorylation site (Y390) abolishes agrin-induced anchoring of mutant AChR and also impairs aggregation. In addition, we have recently found that chimeric proteins consisting of CD4 fused to the β

subunit intracellular loop are sufficient for clustering and that this is dependent on Y390 phosphorylation. In contrast, CD4- δ loop chimeras were not clustered. These findings suggest that phosphorylation of the β subunit regulates protein interactions involved in receptor localization, whereas phosphorylation of the δ loop may serve other functions. Indeed, the tyrosine-phosphorylated δ subunit of Torpedo AChR mediates binding of the adaptor protein, Grb2, via its SH2 domain, consistent with a signaling role (Colledge and Froehner, 1997). Here, we have addressed the mechanism of their phosphorylation and how it is mediated by rapsyn.

Using a simplified heterologous cell system, we find that MuSK induced phosphorylation of both β and δ subunits in a rapsyn-dependent fashion. Moreover, as in muscle (Meier et al., 1995), we observed significant basal phosphorylation of δ and a more pronounced MuSK-induced phosphorylation of β. Despite the basal phosphorylation of δ and its YxxL motif that could allow recruitment of adaptor proteins or kinases (Swope and Huganir, 1994; Fuhrer and Hall, 1996), we found that mutation of Y393 did not inhibit phosphorylation of β. Similarly, mutation of β Y390 did not affect δ phosphorylation. Thus, MuSK-induced phosphorylation of the two subunits is not sequential, with phosphorylation at one site recruiting a kinase that then phosphorylates the second subunit; rather, phosphorylation of the two subunits occurs in parallel.

MuSK-induced phosphorylation of the AChR β and δ subunits involves an intervening cytoplasmic tyrosine kinase (Fuhrer et al., 1997), whose activity and/or localization appears to be rapsyn-dependent (Mohamed and Swope, 1999; Mittaud et al., 2001). Indeed, agrin-induced phosphorylation of the AChR is significantly decreased in rapsyn null myotubes (Apel et al., 1997; Mittaud et al., 2001) and rapsyn activates

cytoplasmic kinases in heterologous cells (Qu et al., 1996; Mohamed and Swope, 1999). By assaying a series of rapsyn deletion mutants, we show that the rapsyn C-terminus is necessary and sufficient for rapsyn's ability to activate kinases and increase cellular phosphorylation. Deletion of aa 366-412 abolished rapsyn-induced phosphorylation, and conversely, the C-terminal half of rapsyn (aa 212-412) was sufficient to induce phosphorylation. Together, this implicates a C-terminal region encompassing the Zn^{++}-binding RING-H2 domain (Froehner, 1991; Scotland et al., 1993) and C-terminal 10 aa's containing a consensus serine phosphorylation site (aa 406) (Froehner, 1991). The RING domain has been previously implicated in binding to β-dystroglycan (Bartoli et al., 2001) but no function has yet been ascribed to the extreme C-terminus (Han et al., 1999). These findings suggest that rapsyn facilitates AChR phosphorylation by regulating tyrosine kinases via its C-terminal domains, and consistent with this, we find that deletion of just the RING domain abolishes MuSK-induced phosphorylation of the AChR β subunit.

While our findings define the rapsyn domains required for kinase interaction, the identity of the cytoplasmic tyrosine kinase remains unclear. One candidate is src family kinases, which are associated with the AChR (Swope and Huganir, 1993, 1994; Fuhrer and Hall, 1996) and are specifically activated following agrin treatment of muscle cells (Mittaud et al., 2001). Src phosphorylates the β and δ subunits in heterologous cells and this is blocked, both by src dominant negative constructs and inhibitors (Mohamed and Swope, 1999). However, agrin induces normal phosphorylation (and clustering) of the AChR in src/fyn double knockout muscle cells (Smith et al., 2001), and studies using src family inhibitors have yielded differing results (Mohamed et al., 2001; Smith et al., 2001). A second candidate is abl/arg kinases, which are localized at the neuromuscular

junction (Finn et al., 2003). Inhibitors of these kinases reduce AChR phosphorylation and clustering (Finn et al., 2003; Mittaud et al., 2004), but it remains unclear whether they directly phosphorylate the receptor. A further possibility is that src and abl kinases combine to phosphorylate the AChR, with their activities being temporally segregated (Mittaud et al., 2004).

Also unclear is whether rapsyn activates or clusters cytoplasmic tyrosine kinases via its C-terminus. Consistent with activation, rapsyn associates with and activates src family kinases in heterologous cells, leading to phosphorylation of AChR and other endogenous cellular proteins (Mohamed and Swope, 1999). Similarly, rapsyn is required for agrin's activation of AChR-associated src family kinases in muscle cells (Mittaud et al., 2001). Rapsyn-induced clustering of kinases could also facilitate AChR phosphorylation, however we were unable to detect rapsyn-associated clusters of src or arg kinases in heterologous cells (data not shown). In addition, no significant concentration of src or abl family kinases has been reported in the postsynaptic membrane at early neuromuscular synapses; abl kinases have been detected only postnatally (Finn et al., 2003). Thus, we favor the idea that rapsyn primarily activates kinases and the requisite kinases may interact with the rapsyn/AChR complex with relatively low affinity or stoichiometry.

Phosphorylation of rapsyn

In COS cells, we found that MuSK alone did not increase global cellular tyrosine phosphorylation and MuSK co-expression with rapsyn did not enhance phosphorylation beyond that induced by rapsyn alone. However, we did observe specific, MuSK-induced phosphorylation of the AChR (discussed above) and rapsyn. Intriguingly, tyrosine

phosphorylation of rapsyn was increased ~3.5 fold by MuSK, suggesting that rapsyn may be a downstream target for phosphorylation in the agrin signaling pathway in muscle. Consistent with this, rapsyn contains several conserved tyrosine phosphorylation consensus sites and rapsyn purified from Torpedo electric organ is tyrosine phosphorylated to some degree (Qu et al., 1996; Balasubramanian and Huganir, 1999; Mohamed and Swope, 1999). The specific site of phosphorylation and its role is unclear, as we found that mutation of Y336 and Y363 did not abolish rapsyn phosphorylation (data not shown) or impair phosphorylation of the AChR. Thus, although it remains to be confirmed that agrin induces rapsyn phosphorylation in muscle, this finding raises the possibility that post-translational modification of rapsyn contributes to AChR clustering and anchoring at the NMJ.

In summary, we find that MuSK-induced phosphorylation of the AChR β and δ subunits occurs in parallel, likely mediated by the same or similar kinases. Moreover, our findings suggest that rapsyn facilitates this phosphorylation by activating tyrosine kinases via its C-terminal domains. These findings imply a common mechanism for agrin-induced phosphorylation of the β and δ subunits, however they likely have distinct functions in AChR localization and function.

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Figure 1. Rapsyn mediates MuSK-induced AChR phosphorylation. AChR was transiently expressed in COS cells alone or with rapsyn and/or MuSK. To assay AChR phosphorylation, both detergent soluble and the insoluble (pellet) fractions of COS cells were immunoblotted with antibodies specific for phosphorylated AChR β and δ subunits (JH-1360 and JH-1358, respectively). The expression level of AChR in each fraction was also determined by blotting for HA-tagged β subunit using anti-HA antibody. When the AChR was expressed alone, we found some basal tyrosine phosphorylation of the δ subunit but minimal phosphorylation of the β subunit. Rapsyn co-expression increased both β and δ subunit phosphorylation, whereas MuSK coexpression had no effect. However, co-expression of MuSK together with rapsyn further increased the levels of β and δ subunit phosphorylation, suggesting that rapsyn mediates MuSK-induced AChR phosphorylation.

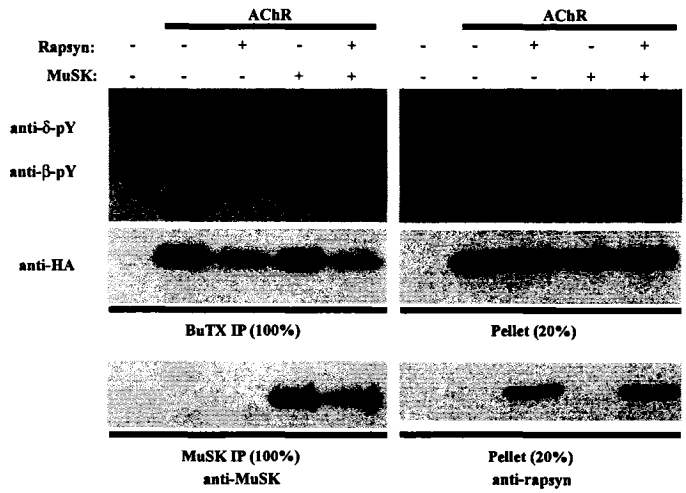

AChR
AChR
Rapsyn: - - + - + - - + - +
MuSK: - - - + + - - - + +
anti-δ-pY
anti-β-pY
anti-HA
BuTX IP (100%)
Pellet (20%)
MuSK IP (100%)
anti-MuSK
Pellet (20%)
anti-rapsyn

Figure 2. Phosphorylation of AChR β and δ subunits are not interdependent. AChR containing either wild type or Y390F β subunit or Y393F δ subunit were transiently expressed in COS cells. (A) After detergent extraction, MuSK/rapsyn-induced phosphorylation of both subunits were determined by immunoblotting the soluble and insoluble (pellet) fraction with antibodies that recognize phosphorylated AChR β and δ subunits (JH-1360 and JH-1358, respectively). The blots were then reprobed with anti-HA antibody to detect the HA-tagged β subunit and determine the levels of AChR expression. (B) Quantification of AChR β and δ subunit phosphorylation shows that β subunit Y390F mutation did not significantly impair δ subunit phosphorylation (78.25 ± 5.95%, mean ± SEM; n=5), nor did δ subunit Y393F mutation inhibit β subunit phosphorylation (88.87 ± 3.48%, mean ± SEM; n=5). Thus, rapsyn/MuSK-induced phosphorylation of the two sites is not interdependent.

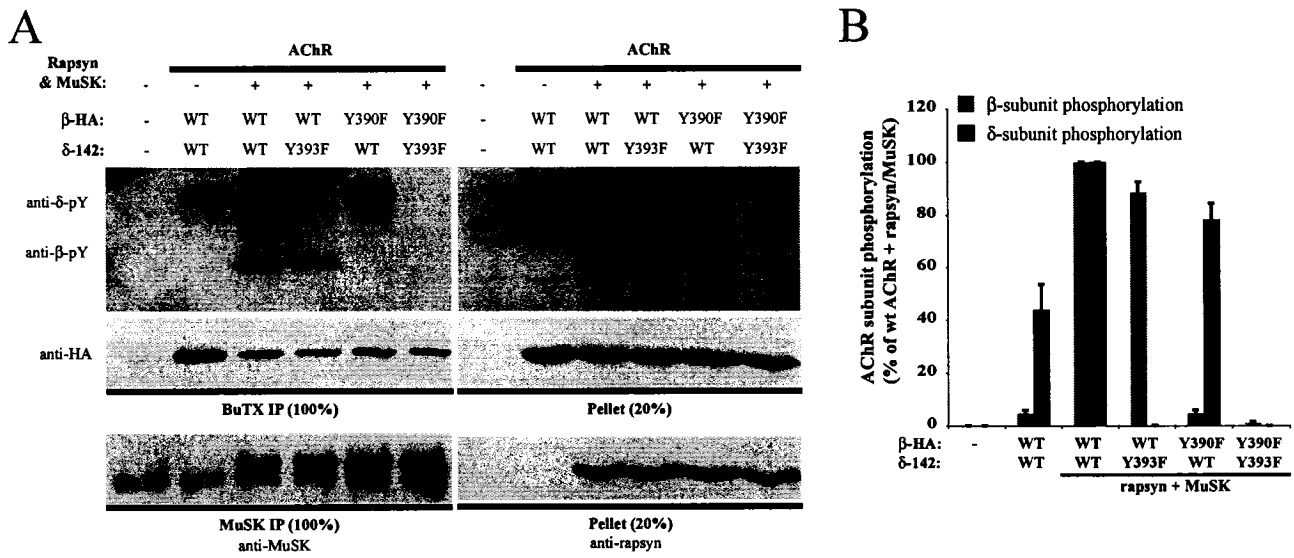

A
Rapsyn & MuSK:
AChR
AChR
β-HA:
δ-142:
anti-δ-pY
anti-β-pY
anti-HA
BuTX IP (100%)
Pellet (20%)
MuSK IP (100%)
anti-MuSK
Pellet (20%)
anti-rapsyn
B
β-subunit phosphorylation
δ-subunit phosphorylation
ACh subunit phosphorylation (% of wt AChR + rapsyn/MuSK)
rapsyn + MuSK

Figure 3. Rapsyn activates cellular tyrosine kinases independent of MuSK. (A) Relative levels of cellular tyrosine kinase activity were estimated by immunoblotting whole cell lysate of COS cells transfected with rapsyn and/or MuSK with monoclonal anti-phosphotyrosine antibodies, 4G10 and PY20. Rapsyn induced a robust increase in tyrosine phosphorylation of cellular proteins. MuSK expression did not result in increased tyrosine phosphorylation and co-expression with rapsyn did not enhance the rapsyn-induced increase in cellular phosphorylation. (B) Quantification of phosphotyrosine immunoblots of COS cell lysates shows that MuSK expression did not enhance the rapsyn-induced increase in cellular tyrosine phosphorylation (123 ± 35% of rapsyn alone, mean ± SEM; n=3) or the basal level of kinase activation (31 ± 23% of rapsyn alone, mean ± SEM; n=3; $p<0.05$). (C) MuSK co-expression significantly increased the tyrosine phosphorylation of rapsyn (360 ± 31% of rapsyn alone, mean ± SEM; n=3; $p<0.05$).

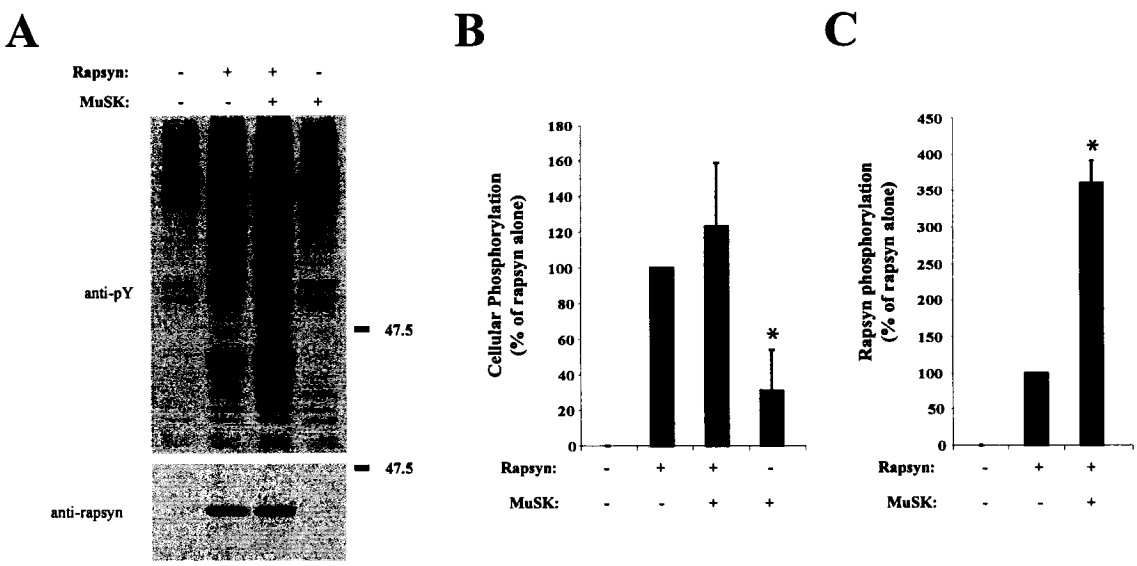

A
Rapsyn: - + + -
MuSK: - - + +
anti-pY
47.5
47.5
anti-rapsyn
B
Cellular Phosphorylation (% of rapsyn alone)
180
160
140
120
100
80
60
40
20
0
*
Rapsyn: - + + -
MuSK: - - + +
C
Rapsyn phosphorylation (% of rapsyn alone)
450
400
350
300
250
200
150
100
50
0
*
Rapsyn: - + +
MuSK: - - +

Figure 4. C-terminal domain of rapsyn is required and sufficient for cellular kinase activation. (A) Schematic representation of rapsyn's functional domains. Rapsyn's N-terminal myristoylation, a fatty acid modification (Musil et al., 1988; Carr et al., 1989), targets the protein to the plasma membrane (Phillips et al., 1991; Ramarao and Cohen, 1998). Rapsyn's 7 tetratricopeptide repeats (TPR's) are implicated in rapsyn self-aggregation (Ponting and Phillips, 1996; Ramarao and Cohen, 1998; Ramarao et al., 2001; Eckler et al., 2005). Rapsyn's C-terminal coiled-coil (C-C) and RING domains are responsible for rapsyn's interaction with AChR (Ramarao and Cohen, 1998) and β-dystroglycan (Bartoli et al., 2001), respectively. (B, D) Phosphotyrosine immunoblot of lysates of COS cells transfected with rapsyn deletion mutants using monoclonal antibodies 4G10 and PY20. (B) Deletion of rapsyn's C-terminal domains abolished its ability to enhance cellular phosphorylation. (C) Quantification of the levels of cellular tyrosine phosphorylation induced by different rapsyn constructs, expressed as a percentage of that induced by wild type rapsyn (FL). Rapsyn (1-365) and (1-319) both failed to increase cellular protein phosphorylation (19.6 ± 12% and 10.9 ± 5.6% of rapsyn FL, respectively; mean ± SEM; n=3; $p<0.01$). (D) Expression of rapsyn C-terminal fragments is sufficient to increase tyrosine phosphorylation of cellular proteins. (E) N-terminal deletion mutants of rapsyn, (158-412)-H6 and (212-412)-H6, both significantly increased phosphorylation of cellular proteins (139 ± 38% and 73 ± 20% of rapsyn FL levels, respectively; mean ± SEM, n=3).

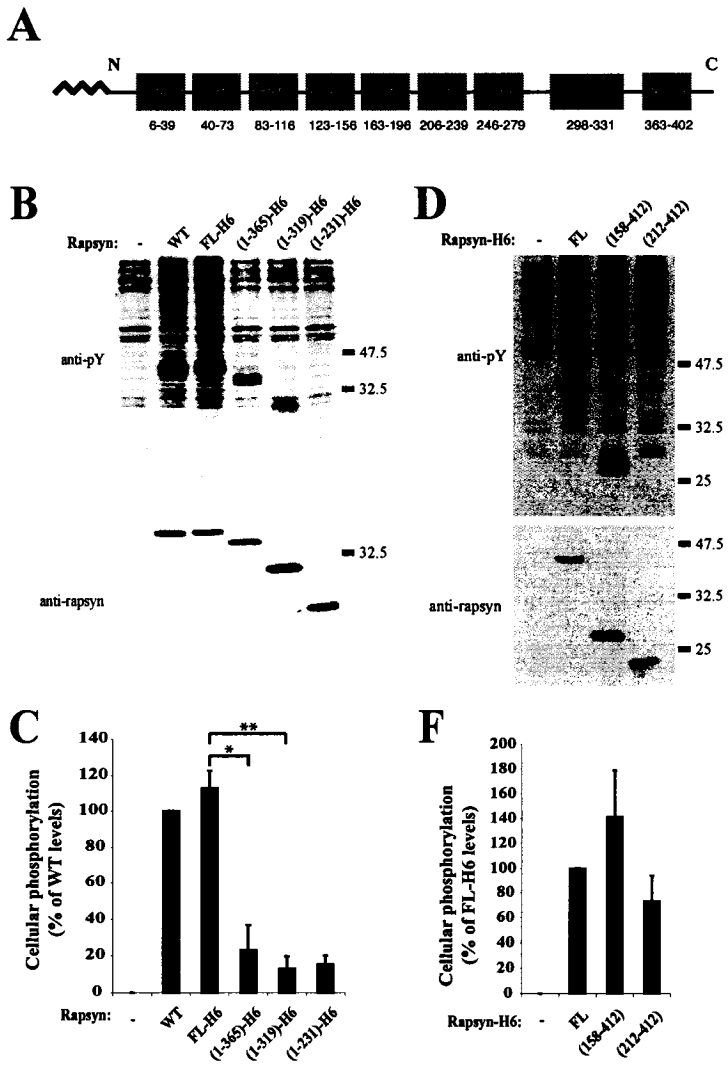

A
N
C
6-39
40-73
83-116
123-156
163-196
206-239
246-279
298-331
363-402
B
Rapsyn:
-
WT
FL-H6
(1-365)-H6
(1-319)-H6
(1-231)-H6
anti-pY
47.5
32.5
anti-rapsyn
32.5
D
Rapsyn-H6:
-
FL
(158-412)
(212-412)
anti-pY
47.5
32.5
25
anti-rapsyn
47.5
32.5
25
C
Cellular phosphorylation
(% of WT levels)
140
120
100
80
60
40
20
0
**
*
Rapsyn:
-
WT
FL-H6
(1-365)-H6
(1-319)-H6
(1-231)-H6
F
Cellular phosphorylation
(% of FL-H6 levels)
200
180
160
140
120
100
80
60
40
20
0
Rapsyn-H6:
-
FL
(158-412)
(212-412)

Figure 5. Rapsyn C-terminus is necessary but not sufficient for MuSK-induced AChR phosphorylation. (A, C) Phosphorylation of AChR β subunit mediated by different rapsyn deletion mutants was determined using a phospho-specific antibody to tyrosine 390 (JH-1360). The blots were then reprobed for AChR β subunit (mAb124), rapsyn (mAb1234), and MuSK (9E10) to determine their expression levels. The C-terminal region of rapsyn is required (A) but not sufficient (C) for AChR β subunit phosphorylation. (B) The relative levels of AChR β subunit phosphorylation mediated by rapsyn (1-365)-H6 and (1-319)-H6 were 17 ±7% and 4.7 ± 3.5% of that seen with rapsyn (FL)-H6, respectively (mean ± SEM; n=3; $p<0.001$). (D) Rapsyn N-terminal deletion mutants, rapsyn (158-412)-H6 and (212-412)-H6, also failed to increase AChR β subunit phosphorylation (2.8 ± 1.1% and 1.8 ± 1.3%, respectively) above background levels (2.5 ± 2.4%; mean ± SEM; n=5; $p<0.001$).

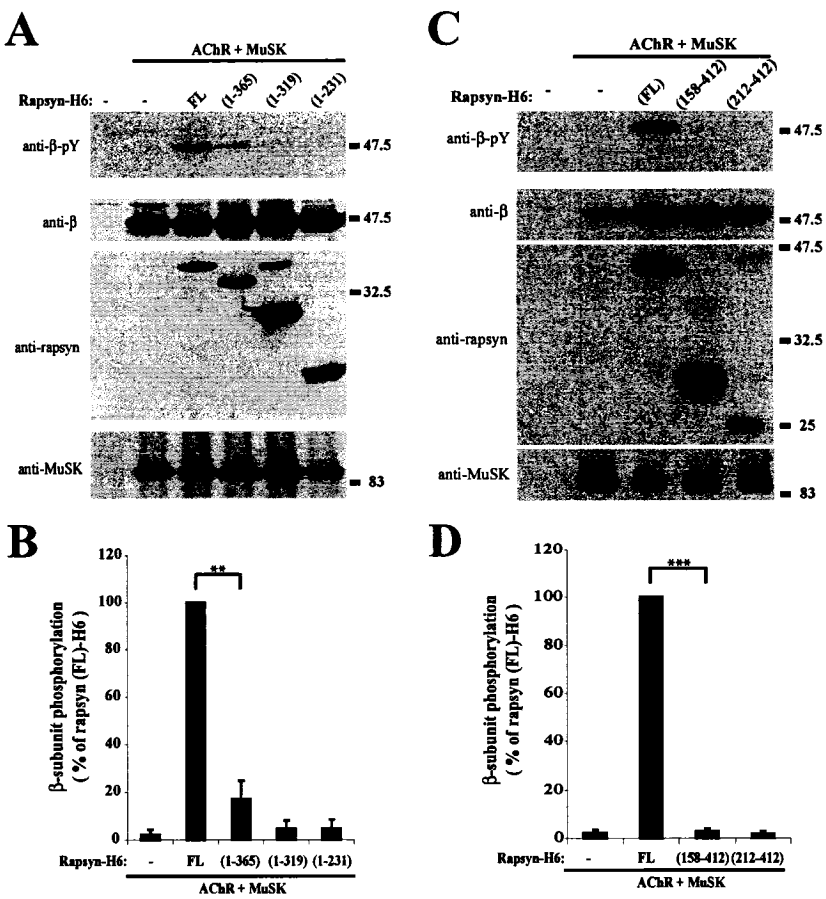
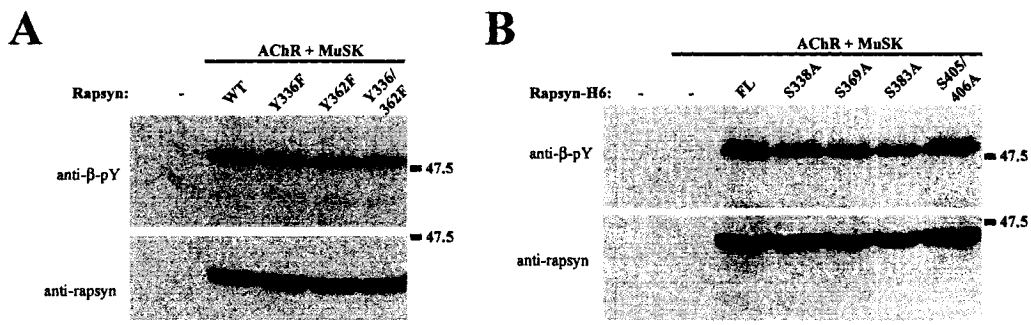

A
AChR + MuSK
Rapsyn-H6: - - FL (1-365) (1-319) (1-231)
anti-β-pY
anti-β
anti-rapsyn
anti-MuSK
47.5
47.5
32.5
83
C
AChR + MuSK
Rapsyn-H6: - - (FL) (158-412) (212-412)
anti-β-pY
anti-β
anti-rapsyn
anti-MuSK
47.5
47.5
32.5
25
83
B
β-subunit phosphorylation (% of rapsyn (FL)-H6)
**
Rapsyn-H6: - FL (1-365) (1-319) (1-231)
AChR + MuSK
D
β-subunit phosphorylation (% of rapsyn (FL)-H6)
***
Rapsyn-H6: - FL (158-412) (212-412)
AChR + MuSK

Figure 6. Rapsyn C-terminal phosphorylation is not required for AChR phosphorylation. Mutation of potential tyrosine (A) or serine (B) phosphorylaton sites found in the C-terminal region of rapsyn did not negatively influence AChR β subunit phosphorylation. AChR β subunit phosphorylation was determined by imunoblotting with JH-1360, an antibody specific to phosphorylated β subunit. (A) Rapsyn tyrosine mutants where residues at positions 336 and 362 were mutated to phenylalanine individually (Y336F, Y362F) or in combination (Y336/362F) induced normal AChR β subunit phosphorylation. (B) Rapsyn serine residues at positions 338, 369, 383, 405, and 406 were replaced with alanine individually or in combination (S338A, S369A, S383A, S405/406A). These serine mutations did not interfere with rapsyn's ability to induce AChR β subunit phosphorylation.


A
AChR + MuSK
Rapsyn:
-
WT
Y336F
Y362F
Y336/362F
anti-β-pY
47.5
anti-rapsyn
47.5
B
AChR + MuSK
Rapsyn-H6:
-
-
FL
S338A
S369A
S383A
S405/406A
anti-β-pY
47.5
anti-rapsyn
47.5

Chapter V

General Discussion

Synaptic localization of AChR at the vertebrate neuromuscular junction is controlled by nerve-derived signaling factors, and involves direct and indirect AChR interactions with several postsynaptic scaffolding and/or cytoskeletal proteins (Banks et al., 2003; Kummer et al., 2006). This process necessitates a muscle-specific scaffolding protein called rapsyn (Gautam et al., 1995). Although rapsyn is thought to directly bind to AChR in order to cluster and anchor the receptor at the synapse, rapsyn's site of interaction on the AChR was not clearly defined. Furthermore, it was not known whether interaction with proteins other than rapsyn also contribute to synaptic localization of AChR. In this thesis, we have investigated the protein interactions that contribute to the high-density accumulation of AChR in the postsynaptic muscle membrane.

In Chapter 2, we mapped rapsyn's site of interaction on the AChR using CD4-AChR subunit loop chimeras. We find that rapsyn interacts selectively with α, β and ε subunit loops, although with different apparent affinities. While the intracellular loops represent the segment of receptor subunits with the least amino acid sequence similarity (~14% similarity; ~5% identity), a conserved α-helical structure mediates the subunit loop-rapsyn interactions; this region, homologous to the β subunit exon10, is conserved between different subunit loops. Interestingly, rapsyn-βE10 interaction in muscle can occur prior to agrin-signaling. This suggests that rapsyn and AChR can interact independent of agrin-signaling, consistent with the preassembled AChR-rapsyn complexes reported previously (Moransard et al., 2003). Rapsyn's interaction with multiple subunit loops provides up to 4 rapsyn binding sites per AChR (2 α, 1 β, 1 ε), however, and recruitment of additional rapsyn to these sites via agrin-signaling might provide a means to enhance receptor clustering and/or anchoring.

Next, in Chapter 3, we used CD4-AChR subunit chimeras to identify a 37 aa motif in the β subunit intracellular loop that is sufficient for agrin-induced clustering in muscle. Overexpression of this β subunit exon9 fragment reduced the number of agrin-induced AChR clusters in transfected myotubes in culture, presumably by competing for a protein important for AChR clustering. Interestingly, the β subunit E9 region contains a conserved tyrosine at position 390 that is phosphorylated in response to agrin and its mutation abolished clustering of CD4-β loop chimeras. Therefore, our findings indicate that this β subunit specific "clustering motif" interacts with a protein critical for normal AChR clustering, and agrin-induced β subunit phosphorylation regulates this interaction.

Interestingly, the amount of rapsyn co-precipitated with AChR is increased with agrin stimulation of cultured myotubes (Moransard et al., 2003). As agrin stimulation also induces robust phosphorylation of AChR β subunit, we tested the hypothesis that rapsyn binds to the β subunit clustering motif in a phosphorylation-dependent manner. We failed to observe detectable interaction between phosphorylated βE9 and rapsyn in heterologous cells. In addition, while there is a close temporal correlation between agrin-induced phosphorylation of β subunit and the amount of rapsyn associated with AChR, mutation of the β subunit Y390F did not alter the agrin-induced increase in rapsyn-AChR interaction. These findings suggest that rapsyn is not the protein that interacts with the β subunit "clustering motif" upon agrin signaling. Moreover, regulated rapsyn-AChR interaction is not regulated by agrin-induced phosphorylation of AChR β subunit, and the enhanced rapsyn to receptor ratio is probably due to additional rapsyn binding to previously vacant subunit α-helices.

In summary, our findings suggest that there are multiple interactions with the AChR that contribute to synaptic localization of AChR aggregates. More specifically, we propose that 3 distinct interactions contribute to various aspects of high-density accumulation of AChR at the muscle endplate: 1) a constitutive rapsyn-AChR interaction, 2) a regulated rapsyn-AChR interaction, and 3) a phosphorylation-dependent protein binding to the 37 aa β subunit "clustering motif."

Preformed AChR-rapsyn complex and agrin signaling

Our findings suggest that rapsyn-AChR interaction (Chapter 2) is mediated by the α-helix found in the large intracellular loops of α, β, and ε subunits. This interaction is unlikely to directly drive AChR clustering as it occurs independent of agrin-signaling and rapsyn associates with non-clustered receptors *in vivo* (Moransard et al., 2003). A recent study estimates that approximately half of rapsyn in muscle is constitutively associated with AChR (Marangi et al., 2001). Interestingly, the remaining pool of free rapsyn is not able to cluster in response to agrin after antibody-induced downregulation of surface AChR (Marangi et al., 2001). As interaction with AChR is required for rapsyn to form aggregates, the pre-assembled AChR-rapsyn complex may be required to initiate agrin-induced receptor aggregates. Alternatively, the constitutive receptor-rapsyn interaction could facilitate agrin-induced AChR clustering to occur rapidly by reducing the amount of additional rapsyn required.

In addition, this constitutive interaction may also have functional significance with respect to agrin signaling cascade. Agrin-induced phosphorylation of AChR, thought to be an important precursor event to receptor clustering, is severely impaired in

rapsyn null myotubes (Apel et al., 1997; Mittaud et al., 2001). In addition, src family kinases, which may phosphorylate AChR (Mohamed and Swope, 1999), are constitutively associated with the receptor and activated by agrin in a rapsyn-dependent manner (Apel et al., 1997; Mittaud et al., 2001). Therefore, agrin signaling could potentially produce phosphorylation of AChR via rapsyn-dependent activation of a kinase all within a preformed protein complex prior to receptor clustering. The fact that agrin-induced receptor phosphorylation precedes detectable clustering by several hours (Ferns et al., 1996) and our findings in Chapter 4, where rapsyn's ability to mediate MuSK-induced AChR phosphorylation closely correlates with its ability to enhance cellular kinase activity, are consistent with this idea.

Agrin-induced clustering of AChR and recruitment of additional rapsyn

The agrin-induced increase in the amount of rapsyn co-precipitated with AChR (Chapter 3) could be due to either increased rapsyn-receptor affinity or recruitment of additional rapsyn to the AChR complex. We favor the latter possibility based on the fact that there are 4 potential binding sites on each AChR that can accommodate additional rapsyn (Chapter 2). In agreement with this idea, a recent study reports that transfection of muscle fibers with tagged-rapsyn resulted in an increased rapsyn:AChR ratio at the NMJ *in vivo*, leading the authors to conclude that additional exogenous rapsyn was targeted to muscle endplate by occupying previously vacant rapsyn binding sites (Gervasio and Phillips, 2005).

The regulated AChR-rapsyn interaction appears to be controlled by agrin-signaling (Moransard et al., 2003); however, it is presently unclear what the precise

mode(s) of regulation is. As our result shows, β subunit Y390 phosphorylation does not regulate AChR-rapsyn interaction despite the close temporal correlation between the two agrin-induced events (Chapter 3). It is likely then that agrin-induced posttranslational modification of another AChR subunit, rapsyn or perhaps an additional protein is responsible for modulating AChR-rapsyn interaction.

One obvious candidate is the tyrosine phosphorylation of receptor δ subunit, which is also tightly correlated with agrin-induced receptor clustering (Mittaud et al., 2001). In fact, agrin-induced phosphorylation of the AChR β and δ subunits display similar time courses and sensitivity to the same pharmacological agents (Mittaud et al., 2001; Mohamed et al., 2001). Posttranslational modification of rapsyn is another plausible way in which its interaction with AChR is enhanced following agrin stimulation. Interestingly, we find that rapsyn is preferentially tyrosine-phosphorylated downstream of MuSK in COS cells (Chapter 4), and rapsyn isolated from *Torpedo* electrocyte is tyrosine phosphorylated (Balasubramanian and Huganir, 1999; Mohamed and Swope, 1999).

Although rapsyn and AChR may interact directly, it is also possible that an intermediary and/or inhibitory protein(s) is responsible for modulating the interaction between the two. Involvement of an inhibitory protein, especially, is consistent with the fact that clustering of AChR in muscle is agrin/MuSK dependent (DeChiara et al., 1996; Gautam et al., 1996; Zhou et al., 1999; Herbst and Burden, 2000; Borges and Ferns, 2001), while in heterologous cells, receptor clustering occurs independent of agrin signaling and requires only rapsyn (Froehner et al., 1990; Phillips et al., 1991). Agrin-induced removal of this proposed inhibitory mechanism might allow additional rapsyn to

interact with AChR and/or oligomerization of rapsyn molecules, eventually aggregating AChR.

Role of β subunit motif in synaptic localization of AChR aggregates

The 37 aa β subunit motif contains a conserved tyrosine (Y390) that is phosphorylated with agrin stimulation and its mutation abolishes agrin-induced increase in receptor anchorage to the cytoskeleton (Borges and Ferns, 2001). Mutation of Y390, however, did not abolish clustering of AChR (Borges and Ferns, 2001). Similarly, while pervanadate, a phosphatase inhibitor, increased phosphorylation of AChR β subunits and receptor anchorage in cultured myotubes, it prevented formation of AChR clusters (Wallace, 1995). A recent report that treatment of cultured myotubes with lithium chloride prevented agrin-induced clustering of AChR while having no negative affects on increased receptor anchorage (Sharma and Wallace, 2003) also demonstrate that the two agrin-induced events can be dissociated. Thus, these findings suggest that clustering and anchoring of the AChR are closely correlated but distinct processes that together efficiently localize AChR clusters at the synapse.

The lack of agrin-induced increase in cytoskeletal anchorage of βY390F AChR (Borges and Ferns, 2001) suggests that the contribution of the β subunit "clustering motif" to the synaptic AChR accumulation is likely to be through receptor anchorage at the synapse. Rapsyn is widely believed to be the AChR anchoring protein based on its ability to interact with synaptic cytoskeletal components (Walker et al., 1984; Apel et al., 1995; Cartaud et al., 1998). Surprisingly, our findings from Chapter 3 strongly indicate that rapsyn is not the binding partner for this "clustering motif." A recent study reports

that in src-/-;fyn-/- myotubes, agrin-induced increase in AChR-rapsyn association occurs normally while receptor anchorage is diminished (Sadasivam et al., 2005), further suggesting that rapsyn is not the receptor anchoring protein. Therefore, it is likely that another protein, perhaps cytoskeletal in nature, is responsible for agrin-induced anchorage of the receptor in muscle. One candidate molecule is α-dystrobrevin. There is a significant increase in the mobility of AChR within the postsynaptic muscle membrane of α-dystrobrevin null animals *in vivo* (Akaaboune et al., 2002). In addition, the metabolic half-life of the receptor in the mutant muscle is dramatically reduced (Akaaboune et al., 2002), and is consistent with the idea that anchorage of the receptor to the cytoskeletal network reduces the probability of receptor endocytosis and degradation (Salpeter, 1999). The frayed appearance of the AChR clusters *in vivo* and the faster dispersal of receptor clusters upon agrin withdrawal *in vitro* (Grady et al., 2000) might also suggest lack of anchoring rather than defects in clustering. Finally, α-dystrobrevin colocalizes with AChR clusters on cultured myotubes, and agrin-induced phosphorylation of AChR β subunit closely mirrors the increase in the amount of α-dystrobrevin co-precipitated with the receptor (Sadasivam et al., 2005). Another candidate AChR-anchoring protein is src (and other members of src-family kinases). While tyrosine phosphorylation of AChR β subunit occurs normally in src-/-;fyn-/- myotubes, there was an overall decrease in AChR anchorage (Sadasivam et al., 2005). Accelerated rate of receptor dispersal upon agrin withdrawal in myotubes lacking src and fyn (Smith et al., 2001) also suggest that src-family kinases are involved in AChR anchorage.

Model for molecular events mediating AChR localization

Based on our findings, we propose a model where at least two distinct receptor interacting proteins are required to produce the agrin-induced AChR localization at the postsynaptic muscle membrane. Prior to MuSK activation (Fig 1A), unclustered AChR exist in a preformed complex with rapsyn and perhaps a cellular tyrosine kinase. Upon agrin stimulation (Fig 1B), preformed rapsyn-receptor complex responds to local agrin/MuSK signaling and produces AChR β subunit phosphorylation by activation of the responsible tyrosine kinase. Subsequently, agrin-signaling produces aggregates of AChR through rapsyn-dependent lateral interactions. In parallel, AChR aggregates are anchored to the synaptic cytoskeleton through binding of the receptor anchoring protein to phosphorylated β subunit Y390. With sustained local agrin-signaling (Fig 1C), formation and subsequent anchoring of additional AChR aggregates will produce high-density accumulation of AChR on the postsynaptic muscle surface.

Efficient assembly of postsynaptic apparatus in the central nervous system also require a combination of lateral clustering and cytoskeletal anchorage of neurotransmitter receptors. For instance, two recent reports support a "two-step" model for assembly of glycinergic synapses where gephyrin-independent clustering of glycine receptors precedes the gephyrin-mediated retention and accumulation of clusters at glycinergic synapses (Meier et al., 2000; Rosenberg et al., 2001). Therefore, identification of proteins and the understanding of molecular processes that modulate synaptic localization of AChR clusters at the vertebrate NMJ will help decipher the mechanisms that govern the high-density neurotransmitter receptor accumulation at central synapses.

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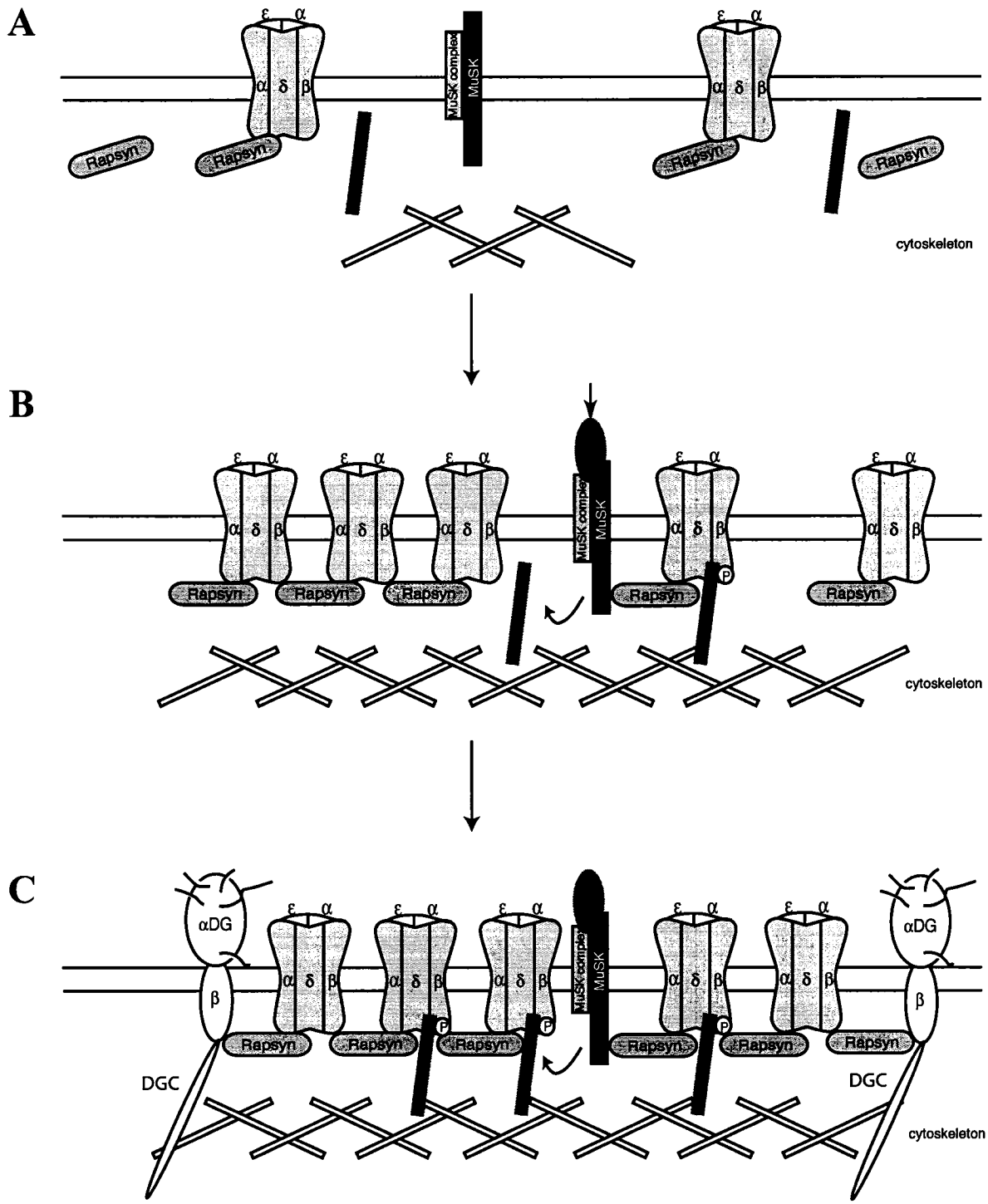
Figure 1. Model for molecular events localizing the AChR in the postsynaptic membrane

Our findings suggest that multiple protein interactions contribute to synaptic localization of AChR. (A) Unclustered AChR and rapsyn are found in a pre-assembled complex prior to MuSK activation. (B) Agrin/MuSK signaling initiates two downstream events that contribute to AChR localization: i) phosphorylation of AChR β subunit and its subsequent interaction with a yet-unidentified protein, and ii) increased AChR/rapsyn interaction that occurs independent of phosphorylation of β subunit Y390. These two protein interactions could localize the AChR by anchoring it to the cytoskeleton and/or clustering it in the membrane. As mutation of β subunit Y390 abolishes agrin-induced anchorage of AChR, we favor the idea that protein binding to the phosphorylated β subunit exon9 region contributes to cytoskeletal anchorage. Moreover, we propose that the increased rapsyn-receptor association may laterally cluster the AChR by binding previously vacant sites and crosslinking the receptor in the postsynaptic membrane. (C) With sustained local agrin/MuSK signaling, the two processes combine to localize AChR at nerve-muscle contact during development. Maturation and stability of the synaptic AChR clusters require additional postsynaptic proteins such as dystroglycans and members of the DGC. For simplicity membrane tethering of rapsyn has been omitted.


A
ε
α
α δ β
MuSK complex
MuSK
Rapsyn
cytoskeleton
B
P
C
αDG
β
DGC

Chapter VI

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